UNIVERSITY OF ALBERTA

Bacteriocin production by *Carnobacterium maltaromaticum* UAL26

by

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A thesis submitted to the faculty of Graduate Studies and Research in partial fulfillment

of the requirements for the degree of **Doctor of Philosophy**

in

Food Science and Technology

Department of Agricultural, Food, and Nutritional Science

Edmonton, Alberta

Fall, 2004

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Your file Votre référence
ISBN: 0-612-95940-6
Our file Notre référence
ISBN: 0-612-95940-6

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DEDICATION

To my parents, Beatrice and Joseph Lee Gursky, for everything they have given to me, including life, love, and much happiness.

ACKNOWLEDGEMENTS

I would like to express my extreme gratitude and love to my parents, Lee and Bea Gursky, for all their support and help. Their influence, motivation, and understanding provided great inspiration and confidence in all my scholastic achievements, culminating in this thesis.

I would like to thank my supervisor, Dr. Lynn M. McMullen, as well as Dr. Michael E. Stiles and Dr. John C. Vederas as my supervisory committee. I would also thank Dr. Peter Sporns and Dr. Randy W. Worobo for their contributions.

A most sincere thanks to everyone who was a source of inspiration to me throughout my life; inspiration is the spirit of the creativity which fuelled my research and powered the writing of this thesis.

I would like to express my thanks to all of the co-workers and friends who helped me complete this stage of my journey, including: Dr. Marco van Belkum for all his help and hours of advice, everyone in the Food Microbiology lab, past and present, Renate Meuser and Wai-Yue Tse from the biotechnology center, Dr. Sylvie Garneau, Dr. Nat Martin, Doug Burr, Dr. Kamaljit Kaur, and Darren Derksen from Dr. Vederas' chemistry lab, and Dr. Randy Whittal from the Department of Chemistry.

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LIST OF ABBREVIATIONS

a.a.: Amino Acid(s)
APT: All purpose Tween
APTNT: All purpose Tween no Tween
ATP: Adenosine triphosphate
ATCC: American Type Culture Collection
A.U.: Activity Units

B.: *Bacillus*
bp: Base pair(s)
Bro.: *Brochothrix*

C.: *Carnobacterium*
CAA: Casamino acids
CAANT: Casamino acids no Tween
Cbn: Carnobacteriocin
CFU: Colony forming units
Ci: Curie(s)
CO_2: Carbon dioxide
CsCl: Cesium chloride

d: Day(s)
Da: Dalton(s)
dATP: Deoxyadenosine triphosphate
dCTP: Deoxycytosine triphosphate
DEPC: Diethylpyrocarbonate
dGTP: Deoxyguanosine triphosphate
DNA: Deoxyribonucleic acid
DNase: Deoxyribonuclease
dNTP: Deoxyribonucleotide triphosphate
DTT: Dithiothreitol
dTTP: Deoxythymidine triphosphate

E.: *Escherichia*
EB: Elution buffer
EDTA: Ethylenediaminetetraacetic acid
Ent.: *Enterococcus*

5': Five prime
fs: Femtosecond(s)

g: Gram(s)
g: Gravity
GRAS: Generally recognized as safe

h: Hour(s)
HCl: Hydrochloric acid
HPLC: High performance liquid chromatography

IP: Induction peptide

K: Degrees(s) Kelvin
kb: Kilobase(s)
kDa: Kilodalton(s)
kJ: Kilojoule(s)
kV: Kilovolt(s)

L.: *Listeria*
l: Litre
LAB: Lactic acid bacteria
Lb.: *Lactobacillus*
LB: Luria Bertani
Lc.: *Lactococcus*
Leu.: *Leuconostoc*
LPS: Lipopolysaccharide

M.: *Micrococcus*
M: Molar
MALDI-TOF: Matrix assisted laser desorption/ionization time-of-flight
mbar: Millibar
μCi: Microcurie(s)
MD: Molecular Dynamics
$MgSO_4$: Magnesium sulphate
min: Minute(s)
μl: Microlitre(s)
ml: Millilitre(s)
μM: Micromolar
mM: Millimolar
μm: Micrometre(s)
mm: Millimetre(s)
mol: Mol(s)
MOPS: 3-(N-morpholino)propanesulphonic acid
mRNA: Messenger RNA
MS: Mass spectrometry

NaCl: Sodium chloride
NaOH: Sodium hydroxide
nM: Nanomolar
nm: Nanometre(s)
NMR: Nuclear Magnetic Resonance
ns: Nanosecond(s)

Ω: Ohm(s)
OD: Optical density

P.: *Pediococcus*
PCR: Polymerase chain reaction
PDB: Protein Data Bank
pM: Picomolar
Ps.: *Pseudomonas*
ps: Picosecond(s)
PTH-a.a.: Phenylthiohydantoin amino acid(s)

RAPD: Random amplified polymorphic DNA
rDNase: Recombinant DNase
RMSD: Root-mean-square deviation
RNA: Ribonucleic acid
RNase: Ribonuclease
ROS: Reactive oxygen species
rpm: Revolution(s) per minute
RPMI: Roswell Park Memorial Institute
RT-PCR: Reverse transcription PCR

S.: *Staphylococcus*
s: Second(s)
Sal.: *Salmonella*
SDS: Sodium dodecyl sulphate
SSC: Saline, sodium citrate
SSPE: Saline, sodium phosphate, EDTA
STE: Sodium chloride, Tris, EDTA
Str.: *Streptococcus*
sup: Supernatant

TBE: Tris, borate, EDTA
TE: Tris, EDTA
TFA: Trifluoroacetic acid
TFE: Trifluoroethanol
3': Three prime
TN150: Tris Sodium Chloride buffer
TRIS: Tris(hydroxymethyl)aminomethane

tRNA: Transfer RNA

U: Unit(s)
UV: Ultraviolet

vol: Volume(s)
vol./vol.: Volume per volume

w./vol.: Weight per volume
w./w.: Weight per weight

1. INTRODUCTION AND REVIEW OF LITERATURE

1.1. General Introduction

1.1.1. Biopreservation of foods by lactic acid bacteria

Preservation of foods may be accomplished through the use of microorganisms, chemical agents, and physical properties (often in combination) that are inhibitory to food spoilage organisms. The methods used to preserve a particular food may also be inhibitory to foodborne pathogens. Preservation of food has been practiced by humankind for millennia. It has proven advantageous to have food that can be stored longer and is less likely to cause food poisoning.

Traditional methods of food preservation by microorganisms include the production of cheese, yoghurt, and other dairy products, fermentation of sauerkraut, and brewing of beer, wine, and spirits. The fermentative processes inherent to these foods is a direct result of the microflora found within. Chemical preservation of foods has long been accomplished by the addition of substances that are inhibitory to the growth of microorganisms. Some examples of traditional food preservation by chemicals include: smoking, curing, pickling, carbonation, or preserving the food with alcohol, sugar, oils or spices. Physical techniques used to preserve foods include cooking, dehydration, refrigeration, freezing, pasteurization, or irradiation. All of these techniques are used to lengthen the amount of time a particular food can be stored before it is consumed.

Preservation of food can result from the growth of lactic acid bacteria (LAB) (Stiles, 1996). Lactic acid bacteria are producers of lactic acid by their metabolism, as well as organic acids, acetaldehyde, ethanol, hydrogen peroxide, carbon dioxide, diacetyl, and other antimicrobial compounds such as reuterin, reutericyclin, and bacteriocins. In addition to modifying the sensory characteristics of the food being fermented, these compounds also contribute to preservation of the food.

Preservation of food is not always advantageous to the prevention of food spoilage or the inhibition of foodborne pathogens. Sometimes the processes used to preserve a food will create a biological niche for food spoilage organisms or foodborne pathogens to grow. In this case, additional hurdles to the growth of these undesirable organisms are often necessary in the production of a particular food. For example, refrigeration is an excellent method to prevent the growth of microorganisms (ICMSF, 1988). However, psychrophilic and psychrotrophic microorganisms are still able to grow at reduced holding temperatures. Problems can arise in refrigerated foods when a foodborne pathogen is able to grow at refrigeration temperatures, as is the case with *Listeria monocytogenes*. The lack of growth of a competitive microflora in some ready-to-eat refrigerated foods leaves a nutrient rich environment in which *L. monocytogenes* is able to thrive (McMullen and Stiles, 1989).

Consumers are becoming cautious of the additives found in the foods they buy. They are increasingly adverse to artificial preservatives, and products that contain these chemicals may be perceived as undesirable. Consumers may also not be conscious of the possibility of the presence of foodborne pathogens in particular foods, and often do not learn proper methods of home food preparation and preservation (Wilcock *et al.*, 2004). This poses a challenge in to food scientists to develop socially accepted methods of preserving foods that are viewed as safe.

Bacteriocins are a type of antimicrobial peptide produced by bacteria that inhibit other bacteria, usually of closely related species to the producer organism (Tagg *et al.*, 1976). They are generally considered to be non-toxic to humans by ingestion because they are naturally found in many foods and have been unwittingly consumed by mankind for thousands of years (Cleveland *et al.*, 2001). Each bacteriocin effectively kills or inhibits the growth of a spectrum of bacteria. Research in our laboratory focuses on the use of bacteriocin-producing LAB species to inhibit food spoilage organisms and foodborne pathogens. In particular, our research involves strains that produce leucocin A, enterocin B, brochocin-C, carnobacteriocins A, BM1, B2, and piscicolin 126. These bacteriocins all belong to the class II non-lantibiotic group and have a relatively narrow activity spectrum. None of these bacteriocins is inhibitory to gram-negative organisms. However, all have the potential for application in the inhibition of food spoilage microorganisms or foodborne pathogens.

Bacteriocin-producing LAB are of interest for their usefulness as natural preservative agents in foods (Stiles, 1996). Either a bacteriocin-producing culture or a preparation of semi-purified or pure bacteriocin could be added to a food. Primarily, the use of bacteriocins as food preservatives is desirable as an alternative to the use of chemical additives and the requirement for certain physical methods of food preservation. Many LAB found in foods are generally considered to be nonpathogenic, can be defined as 'food grade', and can be used for preservation of food. These factors are of benefit in fulfilling regulatory requirements and consumer acceptance.

C. maltaromaticum UAL26 (formerly *C. piscicola* UAL26) was isolated from vacuum-packaged ground beef (Burns, 1987). It produces a detectable amount of bacteriocin when grown on solid agar-based media using the deferred inhibition assay, yet bacteriocin activity can not be detected in the supernatant when *C. maltaromaticum*

UAL26 is grown in broth culture. This is atypical, as bacteriocin production can usually be detected in solid and liquid media. The antibacterial activity produced by *C. maltaromaticum* UAL26 is inhibitory to strains of *L. monocytogenes* (Burns, 1987; Rosario, 2001). The purpose of studying bacteriocin production in *C. maltaromaticum* UAL26 is two-fold. It is of interest as a strain for application in food preservation to reduce or prevent the growth of *L. monocytogenes* in refrigerated food products. It is also of interest because it has an uncharacterized system of control of bacteriocin production with unusual characteristics that have not yet been reported.

1.1.2. Objectives

The objectives of this research were to:

1.) improve production of the bacteriocin(s) produced by *C. maltaromaticum* UAL26;

2.) develop methods to purify sufficient quantities of the bacteriocin(s) produced by *C. maltaromaticum* UAL26 so that they can be identified and genetically characterized;

3.) investigate the basis for control of bacteriocin expression in *C. maltaromaticum* UAL26.

1.2. Literature review

1.2.1. Taxonomy and general description of lactic acid bacteria

Lactic acid bacteria (LAB) belong to the clostridial branch of gram-positive bacteria, and morphologically they are cocci, coccobacilli, or bacilli. They are catalase-negative, non-sporulating and typically have a guanosine plus cytosine (G+C) content below 55 % molar. Lactic acid bacteria are defined as such by their metabolism. To produce energy, LAB require a fermentable carbon source, which is metabolically converted to lactic acid in homofermentative species, or a mixture of lactic and acetic acids and/or ethanol and carbon dioxide in heterofermentative species (Caplice and Fitzgerald, 1999). They can be facultative anaerobes (including microaerophilic) or anaerobes (including aerotolerant anaerobes). Although current and developing molecular methods for taxonomic identification continue to make classification of LAB difficult, they also clear the way for the introduction of a comprehensive and consensus polyphasic classification system (Vandamme *et al.*, 1996).

With reclassifications taken into account, the primary species in the group of LAB include: *Streptococcus*, *Lactococcus*, *Lactobacillus*, *Carnobacterium*, *Enterococcus*, *Leuconostoc*, *Pediococcus*, *Oenococcus*, *Tetragenococcus*, *Vagococcus*, and *Weissella* (Stiles and Holzapfel, 1997).

Lactic acid bacteria are found in a wide variety of environments. They are commonly isolated from a variety of foods, including meat and meat products, dairy products, fermented beverages (including beer and wine), fruit (mashes) and vegetable fermentations, breads (sourdough), sauces and marinades, a multitude of cultural traditional foods, and certain grain and crop products (sugar). They can grow in these foods and be the cause of either spoilage or preservation. Lactic acid bacteria may also be added to foods as starter cultures. They are also commonly found on plants, in soil, sewage, manure, and water. As well, LAB have been isolated from the oral cavity and intestinal tract and the urogenital tract of humans and other animals (Hammes *et al.*, 2002).

1.2.1.1. The genus *Carnobacterium*

Initially classified as the 'atypical' lactobacilli, *Carnobacterium* was deemed to be a separate genus (Collins *et al.*, 1987) based on biochemical and physiological characteristics that set it apart from the lactobacilli. Some of the 'atypical' characteristics of *Carnobacterium* include: growth at pH 9.0, no growth at pH 4.5 or on acetate agar at pH 5.4, and production of the L(+) isomer of lactic acid as the major endproduct of carbohydrate metabolism (>95%). They have been classified as heterofermentative organisms due to the production of a small amount of acetate, formate, and CO_2 under certain growth conditions (Hammes *et al.*, 2002). Carnobacteria are most often linked to the lactobacilli, even though they are phylogenetically more closely related to the genera *Enterococcus* and *Vagococcus* (Stiles and Holzapfel, 1997). Despite this, carnobacteria are typically described, with much similarity to the lactobacilli, as gram-positive, non-sporulating bacilli, with most species being non-motile. They are catalase-negative, and do not reduce nitrate. Carnobacteria are psychrotrophic, being able to grow at temperatures as low as 0°C, while no growth is observed at or above 45°C (Collins *et al.*, 1987). Cresol red thallium acetate sucrose inulin (CTSI) agar can be used to selectively isolate *Carnobacterium* spp. (Wasney *et al.*, 2001).

At the time of the creation of this genus, four species were included: *Carnobacterium piscicola* (Shaw and Harding, 1985), *C. divergens* (Holzapfel and Gerber, 1983), *C. gallinarum* and *C. mobile* (Thornley, 1957). These species were differentiated by ribosomal RNA homology (Wallbanks *et al.*, 1990), phenotype, and physiological differences, such as: sugars usable as fermentable energy sources, motility, growth in the presence of toxic heavy metals, and antibiotic sensitivity (Hammes *et al.*, 2002). Four species have subsequently been added: *C. funditum* and *C. alterfunditum*, both of which are motile species most closely resembling *C. mobile* (Franzmann *et al.*, 1991), *C. inhibens* (Joborn *et al.*, 1999), and *C. viridans* (Holley *et al.*, 2002). New molecular techniques are being used to further differentiate species within the genus. These include randomly amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) (Lai *et al.*, 2000), multiplex PCR (Yost and Nattress, 2000), restriction fragment length polymorphism PCR (Kabadjova *et al.*, 2002), and numerical phenetics (Lai and Manchester, 2000). In these studies, the heterogeneity observed in the representatives of the species *C. piscicola* suggests that the genus could be underspeciated in its current delineation; and also that subspecies of this species exist (Lai and Manchester, 2000; Lai *et al.*, 2000).

Carnobacteria can be commonly isolated from meats, poultry, mould-ripened soft cheese (Millière and Lefebvre, 1994), salmonid fish (Ringo *et al.*, 2001), and, in the case of *C. funditum* and *C. alterfunditum*, in Antarctic lakes (Franzmann *et al.*, 1991). More recently, a case report described the isolation of *C. piscicola* from human pus after a traumatic amputation of the right hand while operating an industrial water sawmill (Chmelar *et al.*, 2002).

1.2.1.2. The species *Carnobacterium maltaromaticum*

Carnobacterium piscicola drew its name from two synonymous predecessors, *Lb. piscicola* (Hiu *et al.*, 1984), and *Lb. carnis* (Shaw and Harding, 1985). However, *C. piscicola* shares 100% identity in rRNA sequence with the species *Lb. maltaromicus* (Miller *et al.*, 1974). As *Lb. maltaromicus* was the first name to be scientifically described, *C. piscicola* should more properly be called *C. maltaromicus* (Collins *et al.*, 1991). However, the wide acceptance of this nomenclature has been slow in gaining acceptance. More recently, *C. piscicola* and *Lb. maltaromicus* have been proposed as a novel combination named *C. maltaromaticum* (Mora *et al.*, 2003). Hence, in this thesis this organism is referred to as *C. maltaromaticum*.

Many strains of *C. maltaromaticum* have been isolated from meat and meat products, the most prominent source being unprocessed, refrigerated red meat (Hammes *et al.*, 2002). However, the species name *C. piscicola* was derived from the fact that it is a fish pathogen. *C. maltaromaticum* infects salmonid fish, including rainbow trout (*Salmo gardneri*), cutthroat trout (*Salmo clarki*), and chinook salmon (*Oncorhynchus tshawytscha*) (Cone, 1982; Hiu *et al.*, 1984). Infection causes blistering and lesion formation on the skin surface and sub-cutaneously, and the internal organs become diseased (Hammes *et al.*, 2002), including peritonitis (Humphrey *et al.*, 1987). Additionally, *C. maltaromaticum* is the cause of 'lactobacillosis' septicaemia in these and other fish species (Hiu *et al.*, 1984).

C. maltaromaticum can be phenotypically differentiated from other species of *Carnobacterium* by its ability to ferment mannitol, mellibiose, and α-methyl-D-mannoside. It is generally more resistant to adverse growth conditions than its counterparts within the *Carnobacterium* genus, including resistance to some heavy metal salts, some antibiotics, and conditions of higher acidity (Hammes *et al.*, 2002).

1.2.2. Use of lactic acid bacteria in foods

Lactic acid bacteria are found ubiquitously in fermented foods and food products. They can be found in all four major food groups. LAB have the status of being adventitious microflora and some species are recognized as 'generally recognized as safe', meaning they are considered to be generally safe for consumption and should not pose a threat to human health (Stiles, 1996). Preservation of foods by LAB occurs through utilization of carbohydrates as an energy source to produce metabolites. These metabolites have several effects upon the fermented food into which they are released. They may improve the organoleptic properties of the food by introducing desirable flavour compounds, creating a more favourable aroma, or modifying the texture of the food. As well, LAB fermentation may have other beneficial effects, including increasing the digestibility of a food and adding functional attributes; a property likely to be used in future research, product development, and marketing (Caplice and Fitzgerald, 1999). Addition of functionality may include probiotic effects or the introduction of desirable value-added 'health' compounds such as vitamins and antioxidants (Steinkraus, 1998). Finally, fermentation allows the introduction of metabolites important in maintaining and improving food quality, food safety and extension of shelf life.

Examples of metabolites that may exert these effects include: organic acids (lactic, acetic, propionic), acetaldehyde, ethanol, hydrogen peroxide, carbon dioxide, diacetyl, reuterin, reutericyclin, and bacteriocins. When these compounds are produced by LAB, food quality and shelf life may be improved through the inhibition of spoilage organisms. Food safety may be improved through the inhibition of foodborne pathogens.

1.2.2.1. The genus *Carnobacterium* in foods

Of the known species within the *Carnobacterium* genus, only *C. maltaromaticum* and *C. divergens* are commonly reported to be isolated from food. These two species are most commonly found in meats, including beef (Buchanan and Klawitter, 1992a; Borch *et al.*, 1996), pork (Grant and Patterson, 1991; Borch *et al.*, 1996), chicken (Grant and Patterson, 1991), and fish (Stoffels *et al.*, 1992a; Leisner *et al.*, 1994; Duffes *et al.*, 1999b). They are isolated from food stored, processed, or packaged under the following conditions: raw (Holzapfel and Gerber, 1983; Buchanan and Klawitter, 1992a), chill-stored (Duffes *et al.*, 1999b; Nilsson *et al.*, 2002), vacuum packaged (Hastings *et al.*, 1996; Duffes *et al.*, 1999b), or modified-atmosphere packaged products (Grant and Patterson, 1991). Additionally, *C. viridans* has been isolated from chill-stored vacuum packaged bologna (Holley *et al.*, 2002). While the original habitat of *Carnobacterium* is unknown, species may also be found in other foods, including various French soft cheeses (Millière and Lefebvre, 1994; Herbin *et al.*, 1997). Anaerobic, reduced oxygen, or microaerophilic environments (as in vacuum packaging or modified atmosphere packaging) and refrigeration create environmental conditions for carnobacteria to flourish due to their anaerobic metabolism and psychrotrophic nature. These species can act as spoilage organisms because they can contribute to development of off-odours and flavours, even though they are desirable in many products because they produce organic acids which aid in preservation (Borch *et al.*, 1996). An aerobic atmosphere in meat packaging can lead to the production of acetoin (sweet, cheesy off-odour) by carnobacteria, and the formation of hydrogen peroxide by *C. divergens*, which results in green discolouration of meat. *Carnobacterium* spp. have also been noted for production of buttery, acidic, and sulphurous off-odours (Borch *et al.*, 1996).

1.2.2.2. *Carnobacterium maltaromaticum* in foods

Carnobacterium spp. flourish in vacuum-packed fish products. The species *C. maltaromaticum* has been isolated from cold smoked salmon (Pilet *et al.*, 1995; Duffes *et al.*, 1999b; Nilsson *et al.*, 1999), and studied for its impact on sensory characteristics. Spoilage of cold smoked salmon occurs rapidly, because it is only minimally preserved by salting and smoking, and therefore it is highly perishable and has a very short shelf life (Stohr *et al.*, 2001). Spoilage and sensory modification of cold smoked salmon products is primarily due to LAB and some gram-negative species, although the process of spoilage is unclear, because not all species present in smoked salmon contribute to spoilage (Leroi *et al.*, 1998; Stohr *et al.*, 2001). *C. maltaromaticum* produces mostly buttery odours, with occasional plastic and rubbery odours (Stohr *et al.*, 2001). This has been attributed to the production of 2,3-butanedione (diacetyl) and 2,3-pentanedione; volatile compounds which have a characteristic odour of butter when in pure form (Joffraud *et al.*, 2001). This buttery odour can be detected when *C. maltaromaticum* grows in meat products (Borch *et al.*, 1996) and broth. This is also likely the odour from which the previously used species name (*C. maltaromicus*) stems (Miller *et al.*, 1974). The sensory effects produced by *C. maltaromaticum* in cold-smoked salmon are not considered to be overt spoilage, because samples inoculated with *C. maltaromaticum* fall into a statistical grouping with control samples that had no sensory changes (Joffraud *et al.*, 2001; Stohr *et al.*, 2001). In fact, samples containing *C. maltaromaticum* are usually not regarded as spoiled, because the odours produced by it are usually not regarded as unpleasant. High numbers of *C. maltaromaticum* inoculated into salmon products do not affect sensory quality (Leroi *et al.*, 1996; Paludan-Muller *et al.*, 1998; Nilsson *et al.*, 1999; Joffraud *et al.*, 2001; Stohr *et al.*, 2001).

1.2.3. Antibacterial activity of lactic acid bacteria

It is widely known that LAB that grow in foods have the ability to inhibit spoilage and pathogenic microorganisms. This occurs through a variety of mechanisms, centering on competitive inhibition. LAB species that grow in a particular food exhibit strong forces that promote their own growth and inhibit the growth of competitors. This is achieved not only by physically occupying space available for growth, but also through chemical and biological processes. Through their metabolism, LAB produce a variety of metabolic products, many of which are exported as compounds required in the extracellular medium or secreted as waste products. Often, these endproducts are compounds that become toxic to cellular growth at a particular concentration. While they may be inhibitory to the producer strain, they also serve to discourage the growth of competitors. This usually results in preservation of the food product. Metabolic endproducts produced by LAB that have an effect of preservation include: carbon dioxide, hydrogen peroxide and other reactive oxygen species (ROS), organic acids, acetaldehyde, ethanol, and diacetyl (Holzapfel *et al.*, 1995; Caplice and Fitzgerald, 1999). These compounds are 'metabolic waste' products: inhibitory substances that are secreted out of the cell into the surrounding medium to maintain the internal stability of the cytoplasmic environment, thereby prolonging growth and survival. They are catabolic products of fermentation. As these compounds are usually toxic to the producer as well as competitors, they eventually become self-inhibiting once a critical concentration in the growth medium is exceeded.

Carbon dioxide is formed from heterolactic fermentation of hexoses, and exerts its antimicrobial effect against those microorganisms which cannot survive in a reduced-oxygen or anaerobic environment. This may be directly inhibitory in the case of strict aerobes (Holzapfel *et al.*, 1995), or may allow anaerobic organisms to outcompete facultative aerobes if available oxygen becomes scarce. Carbon dioxide also reduces permeability when it accumulates in cell membranes, inhibits enzymatic decarboxylation reactions, and will reduce intra- and extracellular pH through the formation of carbonic acid when in solution (Eklund, 1984).

Hydrogen peroxide is bactericidal depending on various intrinsic factors such as pH and temperature (Juven and Pierson, 1996), and exerts its effect by oxidative damage to DNA (Ananthaswamy and Eisenstark, 1977; Imlay and Linn, 1988). More specifically, it causes breakage of the DNA phosphate backbone, preventing DNA replication and the release of nucleotides (Freese *et al.*, 1967). Hydrogen peroxide and hydroxyl radicals are formed from the superoxide anion (White, 1995). Together, these three chemical species exert powerfully damaging oxidative effects upon cellular macromolecules, including oxidizing membrane lipids (thus increasing membrane permeability) or sulphydryl groups of proteins (especially critical enzymes or co-enzymes) (Haugaard, 1968), and causing oxidative stress on DNA, including reaction with thymine or guanine residues (Morris, 1976; Byczkowski and Gessner, 1988; Di Mascio *et al.*, 1989). Many microorganisms are able to negate the inhibitory effects of hydrogen peroxide, superoxide anions, and hydroxyl radicals enzymatically through the use of catalases, peroxidases, and other ROS scavenging enzymes (Archibald and Fridovich, 1981; Condon, 1983; Condon, 1987; Moradas-Ferreira *et al.*, 1996). Gram-negative organisms are intrinsically more resistant to ROS because the lipopolysaccharide layer of the outer membrane helps to trap these molecules and sequester them outside of the cell. However, other mechanisms of defense, including enzymatic inactivation, may not be sufficient for ROS tolerance in certain gram-negative organisms, such as *Escherichia coli* and *Pseudomonas* spp. in food products such as milk (Rolfe *et al.*, 1978). Peptidoglycan is unable to act as a barrier to ROS, and it does not provide the same protection to gram-positive organisms as the LPS layer provides to gram-negative organisms (Dahl *et al.*, 1989). Overall, ROS show variable inhibitory activity among LAB species. Some, but not all, lactococci are inhibited by hydrogen peroxide (Condon, 1987) at a concentration of 0.2 μmol/ml, and are killed by concentrations of 1.5 μmol/ml (Anders *et al.*, 1970). Other lactococci make use of oxidases and peroxidases to ameliorate the effects of hydrogen peroxide. Lactobacilli, on the other hand, are generally not as susceptible to hydrogen peroxide (Gregory and Fridovich, 1974; Yousten *et al.*, 1975; Condon, 1983), because they make use of other ROS defense mechanisms (Archibald and Fridovich, 1981; Condon, 1983; Murphy and Condon, 1984).

Organic acids, primarily lactic (from homofermentative and heterofermentative LAB) and acetic acid (produced by heterofermentative LAB only), and occasionally benzoic (from LAB that can metabolize hypuric acid) (Holzapfel *et al.*, 1995), propionic, and formic acids, reduce the pH of the surrounding medium. They also reduce the intracellular pH, because the undissociated form of the acid is able to cross the cell membrane. Once inside the cell, the acid spontaneously dissociates due to the higher internal physiological pH, and may inhibit growth through alteration of intracellular pH and interference with the membrane potential (Salmond *et al.*, 1984), increased concentration of anions leading to osmotic stress (Piard and Desmazeaud, 1991) or toxicity (Eklund, 1985), and inhibition of some critical enzymatic reactions (Accolas *et al.*, 1980; Krebs *et al.*, 1983). Fermentation by LAB can reduce the pH of a growth medium, and this is often sufficient to inhibit the growth of many food spoilage organisms and foodborne pathogens. At a pH of 4.4 (an endpoint to acidification by many LAB, including *Carnobacterium* spp., *Lactobacillus* spp., and *Lactococcus* spp.), gram-negative organisms such as *E. coli*, *Klebsiella pneumoniae*, *Ps. aeruginosa*, *Salmonella typhi*, *Sal. paratyphi*, *Vibrio parahaemolyticus*, and *Proteus vulgaris*, and gram-positive organisms such as *Bacillus cereus*, *Clostridium botulinum*, *Staphylococcus aureus*, and *Brevibacterium linens* are inhibited (Stadhouders and Langeveld, 1966; Accolas *et al.*, 1980; Asperger, 1986). Most bacteria do not possess innate resistance to low pH conditions; however, some species may use mechanisms of acid tolerance that allow survival in extremely acidic environments. Acid tolerance has been most notably observed in the foodborne pathogens *E. coli* O157:H7 (Lin *et al.*, 1996), and *Listeria monocytogenes* (Davis *et al.*, 1996). *E. coli* O157:H7 has been reported to be tolerant to acidity as low as pH 2.5 (Benjamin and Datta, 1995), which is well below the acidification endpoint of LAB fermentations.

Ethanol is a product of heterofermentative metabolism. At sufficient concentrations, it has the antimicrobial effect of solubilization of lipids, which compromises the integrity of the cellular membrane. It can also denature protein structure. Ethanol may effectively killing or inhibit of foodborne pathogens such as *L. monocytogenes* (Oh and Marshall, 1993; Barker and Park, 2001) *S. aureus*, *Sal. enterica* serovar Choleraesuis, *Ps. aeruginosa*, or *E. coli* O157:H7 (Jordan *et al.*, 1999; Rutala *et al.*, 2000), although the concentration of ethanol required for inhibition in foods may be much higher than that produced by LAB fermentations.

Acetaldehyde may be produced by certain LAB species that utilize threonine aldolase, which enzymatically converts threonine into acetaldehyde and glycine (Accolas *et al.*, 1980). Acetaldehyde becomes inhibitory at a concentration of 10 μg/ml, and can reduce the population of species such as *Lc. lactis* and *Str. thermophilus* by as much as 10^{-1} at 100 μg/ml concentrations (Kulshrestha and Marth, 1975). Growth of the pathogens *S. aureus*, *Sal. enterica* serovar Typhimurium, and *E. coli* is also inhibited by concentrations of acetaldehyde of 10 to 100 μg/ml (Kulshrestha and Marth, 1975). Cellular division of *E. coli* is inhibited by 44 μg/ml of acetaldehyde (Egyud, 1967). Comparably, up to 25 μg/ml of acetaldehyde may be found by natural fermentation of yoghurt, primarily produced by *Lb. bulgaricus* (Accolas *et al.*, 1980).

Diacetyl is produced by many LAB that can metabolize citrate (Cogan, 1980; Lindgren and Dobrogosz, 1990). Gram-positive organisms are generally more resistant to diacetyl than are gram-negative organisms, yeasts, or moulds. Lactic acid bacteria are bacteriostatically inhibited by concentrations exceeding 350 μg/ml, while slight bactericidal inhibition of gram-negative organisms begins at 100 μg/ml, and near total bactericidal inhibition is observed at 200 μg/ml (Jay, 1982; Motlagh *et al.*, 1991). *Lc. lactis* ssp. *lactis* biovar. *diacetylactis* produces an average of 4 μg/ml diacetyl during fermentation (Cogan, 1980), which is below the concentration required for inhibition of either gram-positive or gram-negative bacteria.

In most food fermentations, ethanol, acetaldehyde, and diacetyl may not be present in high enough concentrations individually to be inhibitory (Caplice and Fitzgerald, 1999). In combination, concentrations of these compounds can be a hurdle to the survival and growth of both food spoilage organisms and foodborne pathogens (Ross *et al.*, 2002). These compounds may also act synergistically with each other or other inhibitory factors (Gilliland, 1985).

Compounds such as reuterin (Axelsson *et al.*, 1989), reutericyclin (Ganzle *et al.*, 2000; Holtzel *et al.*, 2000), and bacteriocins are metabolically synthesized, often during the later phases of culture growth, and actively or passively exported from the cell into the surrounding medium. These compounds are usually toxic to their producers; however, the producer organisms may have specific methods of preventing or limiting self-toxicity. In the case of bacteriocins, a gene encoding a bacteriocin immunity protein is usually required. Notably, while resistance to reuterin has not been observed (Dobrogosz *et al.*, 1989), resistance to bacteriocins is well documented (Bouttefroy and Millière, 2000; Eijsink *et al.*, 2002; Gravesen *et al.*, 2002).

Reuterin and reutericyclin are both produced by *Lb. reuteri*. Reuterin (3-hydroxypropionaldehyde; Figure 1.1) is converted enzymatically from glycerol or glyceraldehyde, either of which is required in addition to glucose in the growth medium of *Lb. reuteri* for the production of reuterin. Reuterin is present as a mixture of its monomeric, hydrated monomeric, and cyclized dimeric forms (Talarico and Dobrogosz, 1989). It has a very wide spectrum of activity, including gram-positive and gram-negative bacteria, yeasts and moulds, protozoa, and viruses (Axelsson *et al.*, 1989; Chung *et al.*, 1989). A concentration threshold of 15 to 30 μg/ml inhibits gram-negative bacteria, most gram-positive bacteria, fungi, and protozoa, while a concentration of 60 to 150 μg/ml is required to inhibit LAB, including *Lb. reuteri* (Chung *et al.*, 1989). Reuterin likely exerts its antimicrobial activity by inhibiting ribonucleotide reductases (Dobrogosz *et al.*, 1989).

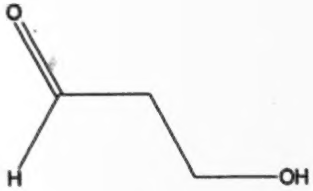

Figure 1.1. The structure of reuterin.

Reutericyclin is a tetramic acid antibiotic with net negative charge, and strong hydrophobicity (Figure 2.2). It is only active against some gram-positive organisms, because it is unable to cross the outer membrane of gram-negative organisms (Ganzle *et al.*, 2000).

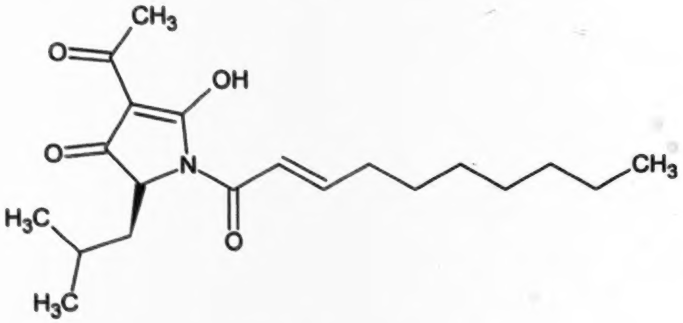

Figure 1.2. The structure of reutericyclin.

1.2.3.1. Bacteriocins of lactic acid bacteria

The antibacterial activity of bacteriocins has been observed since the late 19th century (Florey and Jennings, 1949). This activity was defined more specifically when it was discovered that certain bacteria produce proteinaceous compounds inhibitory to related strains (Rogers and Whittier, 1928; Whitehead, 1933), and the specific term 'bacteriocin' was coined more than fifty years ago (Jacob *et al.*, 1953). While most early research and literature on the subject dealt with the bacteriocins of gram-negative bacteria (primarily the colicins of *E. coli*), a greater surge of research emerged with the discovery (Rogers, 1928) and characterization (Gross and Morell, 1971) of the lanthionine-containing antibacterial peptide nisin (Figure 1.3). Nisin is commonly used as the prototypical example of a bacteriocin. Nisin was originally found to be produced by a strain of *Lc. lactis* (Rogers, 1928), but it has since been isolated from a large number of other strains of *Lc. lactis* (Hurst, 1981). It has a broad spectrum of activity, including many LAB, as well as spores of *Bacillus* and *Clostridium* spp. (Delves-Broughton *et al.*, 1996).

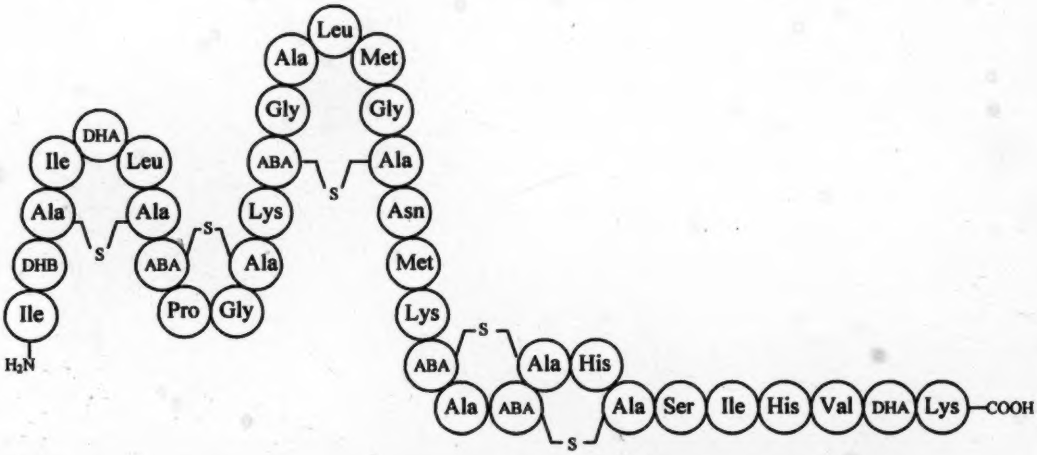

Figure 1.3. The amino acid sequence of nisin. ABA: aminobutyric acid; DHA: dehydroalanine; DHB: dehydrobutyrine; Ala-S-Ala: lanthionine; ABA-S-Ala: β-methyllanthionine.

Nisin has been approved for use as a natural food preservative in over 50 countries worldwide (Delves-Broughton *et al.*, 1996). It is accepted in the European Union (EU) and it has been accepted by the Food and Agriculture Organization (FAO) branch of the World Health Organization (WHO) (Ross *et al.*, 2002). Nisaplin®, a commercial preparation containing 2.5% w./w. nisin, has been produced since 1953, and is used "to control bacterial spoilage in both heat-processed and low pH foods" (Aplin and Barrett, 2003). While nisin cannot be used in certain foods such as meats (Rose *et al.*, 1999; Rose *et al.*, 2002), it is very effective at controlling food spoilage organisms and foodborne pathogens in dairy products and many other foods (Delves-Broughton *et al.*, 1996).

The bacteriocins of gram-positive bacteria have been divided into 4 main classes (Klaenhammer, 1993): I. the lantibiotics (such as nisin): small, membrane-active, heat-stable peptides containing thioether modified amino acids (lanthionine or β-methyl lanthionine) and dehydrated residues; II. non-lantibiotics: small, heat-stable, membrane-active peptides without thioether modified amino acids; III. large, heat labile proteins; and IV. bacteriocins containing other chemical moieties (carbohydrate or lipid).

Although 'small' peptides of the first two groups generally refers to a molecular weight of less than 10 kDa, the third group contains proteins as 'large' as 30 kDa. Heat stability refers to the ability of the bacteriocin to withstand heating temperatures of 100°C for at least 30 min (Klaenhammer, 1993). Class I bacteriocins have been further subdivided into two groups (Jung, 1991): A. elongated peptides with one or more ring structures, and one or more positive charges that permeabilize the target cell membrane; and B. globular peptides with a single ring structure, with a low positive, neutral, or low negative net charge, which inhibit specific enzymes. Thioether rings are introduced to lantibiotic peptides by special protein machinery after the peptide has been synthesized on the ribosome (Kaletta and Entian, 1989; Van der Meer *et al.*, 1993).

While class II bacteriocins do not contain lanthionine or β-methyl lanthionine, they are characterized as typically having a double-glycine motif as the last two residues of a leader peptide, although some make use of the general secretory pathway (using a signal peptide) for export. This class is subdivided into three groups (Klaenhammer, 1993): A. peptides that contain the N-terminal motif YGNGVXC and are active against *Listeria* spp.; B. two-component bacteriocins; and C. peptides with cysteine residues which must be in reduced form for antibacterial activity.

This 'taxonomy' of bacteriocins has been subject to several restructuring alterations and reclassifications in an attempt to amend their organisation according to more current knowledge (Nes *et al.*, 1996; van Belkum and Stiles, 2000), but no single reclassification is yet widely or unanimously agreed upon.

Bacteriocins of LAB exert their inhibitory effect typically by one or both of two mechanisms. The primary mode of action is formation of a pore within the target cell membrane causing efflux of ions and cytoplasmic material, and dissipation of the proton motive force (Wiedemann *et al.*, 2001). The secondary mode of action involves specific binding to a receptor molecule in the cellular membrane. The receptor molecule is usually associated with a critical process necessary for growth or survival. Bacteriocin binding prevents the target cell from normal cellular function through steric interference with the activity of the receptor molecule (Guder *et al.*, 2000; Wiedemann *et al.*, 2001).

Genetic components of the bacteriocins of LAB can be found on plasmid or chromosomal DNA, or both in combination. A bacteriocin operon must contain at least the structural gene, an immunity gene, and a method for export. This export may be through general secretion using a signal sequence at the 5'-end of the structural gene for the *sec*-dependent general secretion pathway (Worobo *et al.*, 1995), or through a specific leader sequence at the 5'-end of the structural gene that tags the bacteriocin for export by genes encoding a specific ATP-binding cassette exporter protein and its accessory protein (Cintas *et al.*, 2001). The bacteriocin operon may also contain other genes, such as a histidine kinase sensory molecule, a response regulator, a gene encoding an induction peptide, or genes required for post-translational modification (Sahl, 1994). For example, the nisin operon contains genes encoding proteins responsible for the formation of its thioether rings (Guder *et al.*, 2000). The genes required for LAB to produce and export functional bacteriocin are generally clustered together, although some 'sharing' of particular genes or their protein products between operons is known: for example, in *C. maltaromaticum* LV17B, the operon for carnobacteriocin BM1 production is chromosomally encoded and does not have transport genes associated with it, and it is hypothesized that it shares this function with genes located in the carnobacteriocin B2 operon, which is located on a plasmid (Quadri *et al.*, 1994). Immunity proteins are usually very specific for their particular bacteriocin, although cross-immunity may also be conferred (Franz *et al.*, 2000). The immunity protein is usually inserted into the cell membrane and may interact with the bacteriocin by binding it and preventing its antibacterial effect, returning it to the external medium, inactivating it (reversibly or irreversibly) or some combination of these effects (Abee, 1995).

A bacteriocin may include residues of one or more amino acids that are necessary in either an exact or close position, and must be either of exact type (e.g. specifically leucine) or of a particular chemical or physical property (acidic, basic, hydrophobic, ring structure) (Zvelebil *et al.*, 1987). These specific amino acids or domains may be active sites where some interaction with the target cell occurs and critical to the activity of the bacteriocin as a whole. Alteration of residues may constitute a drastic change in the physical properties of the bacteriocin. For example, nisin Z differs from nisin A by containing asparagine at residue 27 instead of histidine, resulting in greater solubility in water, better diffusion in solids, and better heat stability, yet there is little difference in spectrum of activity (Hugenholtz and de Veer, 1991).

Control of bacteriocin production in LAB is widely varied. The mechanisms of control may include type of promoter, repression systems, transcription factors, σ-factors, signal molecules, signal receptors (histidine kinases), response regulators, and induction factors (Sahl, 1994). A wide variety of environmental factors may exert an effect on bacteriocin production, including availability of nutrients, cell-density responses (such as quorum sensing and pheromones) (Kuipers *et al.*, 1998; Quadri, 2002), stimulation by the presence of competitor strains (Maldonado *et al.*, 2004), presence of inhibitory factors (Diep *et al.*, 2001), and temperature of growth (Keren *et al.*, 2004).

1.2.3.2. Bacteriocins of *Carnobacterium* spp.

Carnobacteria produce a variety of bacteriocins. To date, bacteriocins produced by carnobacteria reported in the literature have been characterized from *C. maltaromaticum* or *C. divergens*. The only bacteriocin that falls under the class I lantibiotic type is carnocin UI49 from *C. maltaromaticum* UI49 (Stoffels *et al.*, 1992b). Bacteriocins from the class II non-lantibiotic type include piscicolin 126 from *C. maltaromaticum* JG126 (Jack *et al.*, 1996), carnobacteriocin A, BM1, and B2, from *C. maltaromaticum* LV17 (Quadri *et al.*, 1994; Worobo *et al.*, 1994), divergicin A from *C. divergens* LV13 (Worobo *et al.*, 1995), divergicin 750 from *C. divergens* 750 (Holck *et* *al.*, 1996), divercin V41 from *C. divergens* V41 (Metivier *et al.*, 1998), and carnocin H from *Carnobacterium* sp. 377 (Blom *et al.*, 2001). There are no particular unifying traits of immunity, export, genetic organization, or control of production among the bacteriocins from *Carnobacterium* spp., but most exhibit an antibacterial effect against *L. monocytogenes*. Developing ways to use these bacteriocins in foods may prove to be very effective in the control of this important foodborne pathogen, and may contribute to the enhancement of the safety of the food supply.

1.2.3.3. Bacteriocins of *Carnobacterium maltaromaticum*

C. maltaromaticum is inhibitory to the survival and growth of *L. monocytogenes* in foods. In particular, strains of *C. maltaromaticum* have been used to control the growth of *L. monocytogenes* in smoked salmon (Leroi *et al.*, 1996; Duffes *et al.*, 1999a; Nilsson *et al.*, 1999). Other foods in which the antibacterial activity of *C. maltaromaticum* has been studied for its preservative effects include: skimmed milk (Mathieu *et al.*, 1994), UHT milk, canned cooked beef dog food, raw ground beef, irradiation-sterilized raw ground beef, chicken roll, pasteurized crabmeat, canned creamed corn, wieners (Buchanan and Klawitter, 1992b), vacuum packaged cooked chicken (Campos *et al.*, 1997), pork sausages (Roller *et al.*, 2002), and beef steaks (Schöbitz *et al.*, 1999). The bacteriocins that have been characterized from *C. maltaromaticum* include: piscicolin 126, carnobacteriocins A, BM1, and B2, and carnocin UI49.

Piscicolin 126 is produced by *C. maltaromaticum* JG126, which was isolated from spoiled ham. This bacteriocin is a class IIa peptide. It is heat and acid stable, but alkaline labile at a pH greater than 8. It has a spectrum of activity inhibitory to a variety of LAB species, including some foodborne pathogens such as *L. monocytogenes* (Jack *et al.*, 1996).

Carnobacteriocin A is produced by *C. maltaromaticum* LV17, a strain isolated from vacuum packaged meat (Shaw and Harding, 1984). Carnobacteriocin A is plasmid encoded in this strain. *C. maltaromaticum* LV17 was cured of plasmids and the resultant plasmidless strain was named *C. maltaromaticum* LV17C (Ahn and Stiles, 1992). A 49-kb plasmid named pCP49, originating from *C. maltaromaticum* LV17 and containing the carnobacteriocin A operon, was introduced into *C. maltaromaticum* LV17C with as *C. maltaromaticum* LV17A (Ahn and Stiles, 1992). Carnobacteriocin A is a class IIa bacteriocin with a molecular weight of 5052.85 Da. It is characterized as being unstable in acidic conditions of pH 6 or lower, and it is hydrophobic. Its structure shares little homology with other class IIa bacteriocins, but it contains a disulphide bridge between two cysteine residues at amino acid positions 22 and 51. Carnobacteriocin A is active against *P. acidilactici*, *Lb. sakei*, *Ent. faecalis*, *Ent. faecium*, and *L. monocytogenes*.

Carnobacteriocin BM1 is produced by *C. maltaromaticum* LV17, and also by *C. maltaromaticum* LV17B, a strain created by the introduction of the 61-kb plasmid pCP40 into *C. maltaromaticum* LV17C (Ahn and Stiles, 1992). Carnobacteriocin BM1 is a class IIa bacteriocin with a molecular weight of 4524.6 Da. Its genetic elements consist of a structural gene and an immunity gene located on the chromosome. Carnobacteriocin BM1 is co-expressed in *C. maltaromaticum* LV17 with carnobacteriocin B2 (Quadri *et al.*, 1997). Carnobacteriocin B2, another class IIa bacteriocin with a molecular weight of 4969.9 Da, is genetically encoded on the plasmid pCP40. Plasmid pCP40 is necessary for expression of the carnobacteriocin BM1 structural and immunity genes, because it may encode crucial trans-acting factors related to export (Quadri *et al.*, 1995). These two bacteriocins are active against *Lb. plantarum*, *P. parvulus*, *Ent. faecalis*, *Ent. faecium*, and *L. monocytogenes*.

Carnocin UI49 is a class I lantibiotic produced by *C. maltaromaticum* UI49, a strain that was isolated from fish. While the complete sequence of carnocin UI49 has not been elucidated, it reportedly has a molecular weight of 4635.1 Da (Stoffels *et al.*, 1992b). This value, if accurate, makes it the largest bacteriocin known in the lantibiotic family. Carnocin UI49 is active against *Lb. reuteri*, *Lb. sakei*, *Lc. lactis*, and *P. acidilactici*. Strains of *Lc. lactis* that carry the nisin gene cluster have more than 10-fold increased sensitivity to carnocin UI49. This phenomenon has been postulated to be attributable to the use of NisP, a membrane associated protein, by carnocin UI49 as a receptor (Stoffels *et al.*, 1994).

1.2.3.4. Piscicolin 126

Piscicolin 126 belongs to the class IIa bacteriocin group, because it has an intrapeptide disulfide bridge, and contains the YGNGVXC amino acid motif near the N-terminus of the mature peptide. It has a molecular mass of 4416.6 +/- 1.9 Da (Jack *et al.*, 1996). It contains 44 amino acids, with the intrachain disulfide bridge connecting the cysteine residues at positions 9 and 14 (Figure 1.4). It shares the greatest homology with other class IIa bacteriocins, particularly sakacin P (from *Lb. sakei*) and pediocin PA-1 (from *P. acidilactici*); having 75% and 55% identity, respectively (Jack *et al.*, 1996).

Piscicolin 126 is subject to proteolysis by the proteases trypsin, α-chymotrypsin, β-chymotrypsin, protease I, protease XIV, and protease XXIII, and its antibacterial activity is not affected by treatment with catalase, lipase, or lysozyme (Jack *et al.*, 1996).

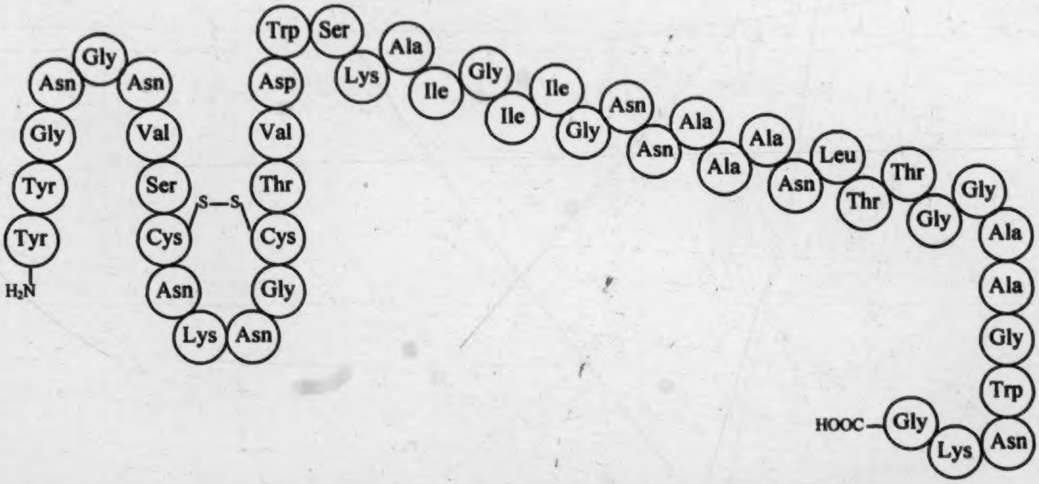
H₂N
Tyr
Tyr
Gly
Asn
Gly
Asn
Val
Ser
Cys
Asn
Lys
Asn
Gly
Cys
Thr
Val
Asp
Trp
Ser
Lys
Ala
Ile
Gly
Ile
Ile
Gly
Asn
Asn
Ala
Ala
Ala
Asn
Leu
Thr
Thr
Gly
Gly
Ala
Ala
Gly
Trp
Asn
Lys
Gly
HOOC
S—S

Figure 1.4. The amino acid sequence of piscicolin 126. S-S: disulphide linkage.

Piscicolin 126 is stable at low pH values, and its antibacterial activity may be destroyed by exposure to alkaline conditions, and more so by alkaline conditions combined with heat (Jack *et al.*, 1996).

Piscicolin 126 is active against numerous gram-positive organisms, including strains of *Brochothrix thermosphacta*, *Carnobacterium* spp., *Ent. faecalis*, *Ent. faecium*, *Lb. curvatus*, *Lb. sakei*, *Leuconostoc dextranicus*, *Leu. mesenteroides*, *P. pentosaceus*, *Str. thermophilus*, *L. grayi*, *L. innocua*, *L. ivanovii*, *L. seeligeri*, and *L. monocytogenes*. Piscicolin 126 does not inhibit the growth of strains of the following gram-positive organisms: *B. cereus*, *B. polymyxa*, *B. stearothermophilus*, *B. subtilis*, *Cl. botulinum*, *Cl. sporogenes*, *Corynebacterium* spp., *Debaryomyces hansei*, *Lb. plantarum*, *Lc. lactis*, *Leu. cremoris*, *L. denitrificans*, *Micrococcus luteus*, *M. varians*, *P. acidilactici*, *S. aureus*, *S. carnosus*, *S. epidermidis*, or strains of the following gram-negative species: *Aeromonas hydrophila*, *Enterobacter aerogenes*, *E. coli*, *Proteus vulgaris*, *Ps. aeruginosa*, *Ps. fluorescens*, *Sal. enterica* serovar Typhimurium, *Sal. enterica* serovar Salford, *Serratia* *marcescens*, or *Yersinia enterocolitica*. Piscicolin 126 is also not active against the yeast *Saccharomyces cerevisiae* (Jack *et al.*, 1996).

Piscicolin 126 reduced the viable counts of *L. monocytogenes* 4A in ham paste stored at 10°C to below the detectable limit (~100 CFU/g) with a starting population of ~1.6 x 10^3 CFU/g. As well, piscicolin 126 inhibited the growth of *L. monocytogenes* 4A for more than 20 d in milk inoculated with 10^2 CFU/ml. Piscicolin 126 also reduced the population of *L. monocytogenes* 4A by as much as 10^5 in milk inoculated with 10^4 to 10^6 CFU/ml. However, at higher inoculum levels, regrowth of resistant *L. monocytogenes* occurred within 24 h (Wan *et al.*, 1997). The addition of piscicolin 126 to milk used to make Camembert cheese containing an initial inoculum of 10^2 CFU/ml of *L. monocytogenes* had a 10^4 lower viable population of the pathogen compared with the control without added piscicolin 126 (Wan *et al.*, 1997). Viable population count and acid production by starter strain bacteria and moulds from the milk and cheese remained unaffected with the inclusion of piscicolin 126. Piscicolin 126 was likely degraded by proteolytic enzymes produced by the starter strains in the Camembert cheese, resulting in the eventual recovery of the *L. monocytogenes* 4A population during cheese ripening (Wan *et al.*, 1997).

Piscicolin 126 can be purified by adsorption onto a variety of food-grade adsorbents, including the commercial products Hi-Sil HOA (PPG Industries, Pittsburgh, PA, U.S.A.), Micro-Cel E, Filter Cel (Celite Corporation, CA, U.S.A.), Celite 521 (Sigma Aldrich, U.S.A.), and titanium dioxide (BDH Chemicals, U.K.). This purification method removes up to 99% of proteinaceous substances from culture supernatants (Wan *et al.*, 1996).

The genetic sequence encoding the mature piscicolin 126 bacteriocin (*pisA*) was cloned into an expression vector in *E. coli* and expressed as a thioredoxin-piscicolin 126 fusion protein (Gibbs *et al.*, 2004). This fusion protein was purified by affinity chromatography and cleaved using cyanogen bromide. Reversed-phase HPLC was used to purify the cleavage product, namely recombinant piscicolin 126. The purified peptide retained the molecular mass, biochemical properties, and antibacterial activity against *L. monocytogenes* 4A of piscicolin 126 (Gibbs *et al.*, 2004). This method of bacteriocin purification resulted in a yield of 26 mg of pure bacteriocin/L, and was useful as an efficient large-scale preparatory method to produce active piscicolin 126 (Gibbs *et al.*, 2004).

Intravenous injections of 2 μg of piscicolin 126 reduced viable counts of *L. monocytogenes* 4A *in vivo* in mice challenged by intravenous injections of up to 10^4 CFU of the pathogen (Ingram *et al.*, 2003). Piscicolin 126 retained antilisterial activity *in vivo* in mice, and was not degraded by proteases in the blood within a minimum 15 min time frame (Ingram *et al.*, 2003). The piscicolin 126 peptide was shown to be non-toxic to the mice by intravenous or intraperitoneal injection (Ingram *et al.*, 2003). However, it was assumed that piscicolin 126 was highly likely to be recognized as foreign antigen by the immune system, and which was expected to produce antibodies to remove piscicolin 126 from the bloodstream (Ingram *et al.*, 2003). As well, it was postulated that piscicolin 126 would not be able to cross the eukaryotic cell membrane without specific modifications for receptor uptake, which limits the potential for use as a replacement for conventional antibiotic therapy (Ingram *et al.*, 2003).

1.2.3.5. Conclusion

Lactic acid bacteria are primarily of interest for their biopreservative effects in food. This is primarily due to their ability to produce antimicrobial compounds, including bacteriocins. Bacteriocins and bacteriocin-producing cultures have the potential to increase the shelf life of foods and decrease the incidence of foodborne disease. *C. maltaromaticum* UAL26 is a lactic acid bacterium that produces bacteriocin activity inhibitory to food spoilage organisms such as *L. sakei*, and foodborne pathogens, such as *L. monocytogenes*, and may be useful as a biopreservative. Use of *C. maltaromaticum* UAL26 as a biopreservative requires elucidation of the genetics of bacteriocin production. However, *C. maltaromaticum* UAL26 does not produce detectable amounts of bacteriocin activity when grown in broth media at 25°C. Evaluating the effect of different growth conditions and media composition may allow isolation and characterization of the bacteriocins produced by *C. maltaromaticum* UAL26. Reverse genetics can then be used to identify the genetic elements encoding the bacteriocin(s) produced by *C. maltaromaticum* UAL26. The genetic elements can then be characterized for their involvement in bacteriocin expression and control of production in *C. maltaromaticum* UAL26.

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2. EFFECT OF MEDIA COMPOSITION AND TEMPERATURE ON BACTERIOCIN PRODUCTION BY *Carnobacterium maltaromaticum* UAL26

2.1 Introduction

New strains of bacteriocin-producing lactic acid bacteria (LAB) are commonly isolated from food sources. Typically, LAB are isolated from a selected food sample and screened for antibacterial activity against a selection of indicator strains. Strains that are bacteriocin-producers are characterized taxonomically, and the bacteriocin is identified. The types of food and number of isolates screened may be large (e.g. 746,533 strains from 112 dairy, meat, fish, and vegetable sources) (Coventry *et al.*, 1997), or small (e.g. 40 strains of LAB isolated from commercial food products and local farms, 49 strains of LAB isolated from commercially available ready-to-eat meat products) (Garver and Muriana, 1993; Amezquita and Brashears, 2002), and may involve either plating for all microorganisms or enrichment for a particular species. Whichever methods are chosen for screening, the goal is usually the same: identification of novel organisms and their bacteriocins to be studied for use in foods.

Bacteriocin-producing LAB are typically screened using assays that demonstrate the inhibitory effect of any antibacterial compound. Use of a range of indicator organisms allows for more efficient screening for bacteriocin production, because bacteriocins are often limited to a narrow spectrum of activity. Two of the most typical methods for detecting and quantifying bacteriocin activity include the spot-on-lawn (critical dilution) assay (Ahn and Stiles, 1990), and the deferred inhibition assay (Tagg *et* *al.*, 1976). Other methods have been reported as effective in the selection of bacteriocin producing organisms (Saucier and Greer, 2001; Somkuti and Steinberg, 2002).

Once a new bacteriocin-producing strain is isolated, it is usually tested for bacteriocin production across a range of growth conditions, including variation in media composition (Parente and Hill, 1992; Nilsson *et al.*, 2002), temperature (Parente and Hill, 1992), and pH of the growth medium (Parente and Hill, 1992). These factors can affect the amount of bacteriocin produced. Characterization of bacteriocins requires sufficient quantity to be used in applications such as protein sequencing and biochemical testing, thus optimization is often done to increase the amount of bacteriocin produced by a culture.

Carnobacterium maltaromaticum UAL26 was isolated from vacuum packaged ground beef (Burns, 1987). It was characterized as producing antibacterial activity through inhibition of the growth of indicator organisms by deferred inhibition tests. This antibacterial activity was confirmed to be proteinaceous in nature by its inactivation when treated with protease enzymes. The spectrum of activity spans a variety of gram-positive bacteria, including species within the genera *Pediococcus*, *Lactobacillus*, *Leuconostoc*, *Enterococcus*, *Bacillus*, *Clostridium*, and *Listeria* (Burns, 1987). Although the inhibition produced by *C. maltaromaticum* UAL26 can be detected using a deferred inhibition assay, antibacterial activity can not be detected in the supernatant by the spot-on-lawn technique.

The starting pH of APT agar plates used to grow *C. maltaromaticum* UAL26 had little effect on the diameter of the zone of inhibition observed in deferred inhibition assays against several indicator organisms within the pH range of 6.5 to 7.5. At pH 6.0

no inhibition is observed, and the producer organism grows poorly from pH 5.5 to 5.0 (Burns, 1987). Temperature of incubation affects bacteriocin production in *C. maltaromaticum* UAL26 when zones of inhibition are measured using a deferred inhibition assay against the indicator strains *Leu. mesenteroides* ATCC 23368 and *Lb. viridescens* ATCC 12706 (Burns, 1987). Attempts to isolate plasmid DNA from *C. maltaromaticum* UAL26 were unsuccessful, leading to the conclusions that this is a plasmidless strain and that the genetic determinants for bacteriocin production in *C. maltaromaticum* UAL26 are chromosomally mediated (Burns, 1987).

Attempts to isolate the antibacterial activity from the supernatant of a liquid culture by ammonium sulphate precipitation or by variations of an agar extraction technique (Barefoot and Klaenhammer, 1983) were unsuccessful (Burns, 1987). Other attempts to purify the antibacterial peptide from *C. maltaromaticum* UAL26 resulted in poor recovery of active bacteriocin, which prohibited isolation and identification of the bacteriocin from other antibacterial components within the culture fermentation (Rosario, 2001). To improve the quantity of bacteriocin produced by *C. maltaromaticum* UAL26, modifications of the growth conditions for production of bacteriocin by *C. maltaromaticum* UAL26 included media supplementation, shaking of the culture, time of bacteriocin isolation, increasing the temperature of incubation, alteration of initial pH of the media, and maintenance of pH of the media during growth (Rosario, 2001). These modifications did not result in recovery of increased amounts of bacteriocin from the culture supernatant. Various purification methods were also attempted, yet an insufficient concentration of bacteriocin, combined with the presence of traces of other antibacterial components intrinsic to the media, prevented purification of the bacteriocin (Rosario, 2001).

The objective of this research was to find suitable growth conditions for *C. maltaromaticum* UAL26 that would result in recovery of the bacteriocin in quantities sufficient for purification.

2.2. Materials and Methods

2.2.1. Bacterial strains and growth media

C. maltaromaticum UAL26 was acquired from the University of Alberta Lactic Acid Bacteria collection (University of Alberta, Edmonton, AB). Stock cultures of *C. maltaromaticum* UAL26 were maintained at -70°C in All Purpose Tween broth (APT; Difco, BD Diagnostics, Sparks, MD) with glycerol (20% vol./vol.; Fisher Scientific, Edmonton, AB). Cultures of *C. maltaromaticum* UAL26 used in this study were prepared by inoculation of cells from frozen stock culture into broth media. Cultures were subcultured at least once more at an inoculation volume of 1% vol./vol. before use in experiments.

Bacteriocin production in *C. maltaromaticum* UAL26 was evaluated in various media, including APT, Casamino acids (CAA) (Hastings *et al.*, 1991), APT without Tween 80 (APTNT), CAA without Tween 80 (CAANT), and a defined medium named Roswell Park Memorial Institute-1640 Auto mod (RPMI; Sigma-Aldrich, Saint Louis, MO). Media variations not including Tween 80 were made using the formulation of the manufacturer, except for the exclusion of the Tween 80.

Cultures used as indicator organisms in the deferred inhibition assay and their sources are listed in Table 2.1. All lactic acid bacteria were stored and grown in APT as described above for *C. maltaromaticum* UAL26. *Brochothrix thermosphacta* and *Listeria monocytogenes* were stored as described above for *C. maltaromaticum* UAL26, and grown in APT broth at incubation temperatures of 30°C and 37°C, respectively.

Table 2.1. Cultures used as indicator organisms in the deferred inhibition assay.

Indicator organism	Reference or Source
Carnobacterium maltaromaticum UAL26	UALABCC*
Carnobacterium maltaromaticum LV17	(Shaw and Harding, 1984)
Carnobacterium divergens LV13	(Shaw and Harding, 1984)
Brochothrix campestris ATCC 43754	ATCC**
Brochothrix thermosphacta ATCC 11509	ATCC
Enterococcus faecalis ATCC 7080	ATCC
Enterococcus faecium ATCC 19434	ATCC
Enterococcus faecium BFE900	(Franz *et al.*, 1996)
Lactococcus lactis ssp. *lactis* ATCC 11454	ATCC
Lactobacillus sakei 706	(Schillinger *et al.*, 1991)
Lactobacillus sakei 1218	UALABCC
Lactobacillus sakei UAL185	UALABCC
Leuconostoc gelidum UAL187	UALABCC
Leuconostoc mesenteroides Y105	(Hechard *et al.*, 1992)
Pediococcus acidilactici PAC1.0	(Pucci *et al.*, 1988)
Listeria monocytogenes ATCC 15313	ATCC
Listeria monocytogenes CDC 7762	CDC***
Listeria monocytogenes ATCC 43256	ATCC
Listeria monocytogenes HPB 642	Health Canada****
Listeria monocytogenes UAFM 1	UAFMCC*****
Listeria monocytogenes UAFM 15	UAFMCC

* UALABCC: University of Alberta Lactic Acid Bacteria Culture Collection
** ATCC: American Type Culture Collection
*** CDC: Centers for Disease Control and Prevention
**** Obtained from E. Daley, Health Canada, Ottawa, ON
***** UAFMCC: University of Alberta Food Microbiology Culture Collection

2.2.2. Antibacterial activity of *C. maltaromaticum* UAL26

L. monocytogenes ATCC 43256 was grown from frozen stock culture in APT broth at 37°C for 24 h, and subcultured at least once before use in experiments as the indicator organism to determine arbitrary activity units (A.U.) of bacteriocin activity in spot-on-lawn assays. Arbitrary activity units were calculated as the reciprocal of the highest dilution in a doubling dilution series that showed a clear zone of inhibition, and are expressed in A.U./ml (Ahn and Stiles, 1990). For the spot-on-lawn assay, 10 µl of each dilution of cell-free (centrifuged at 10,000 x *g* for 5 min), heated (65°C for 30 min) supernatant was spotted onto APT agar plates, and overlayered with 7 ml of soft APT agar (0.75% agar) seeded with a 1% inoculum of *L. monocytogenes* ATCC 43256. Plates were incubated at 37°C for 24 h, and zones of inhibition were measured and A.U./ml calculated.

The indicator strains used to measure the antibacterial activity and spectrum of activity of *C. maltaromaticum* UAL26 by deferred inhibition are listed in Table 2.1. For the deferred inhibition assay, a 1 µl spot of a culture was inoculated onto APT agar plates (1.5% agar), allowed to dry, and incubated until the culture reached maximum population (1 to 12 d) at temperatures that ranged from 1 to 25°C, depending on the experiment. The plate was overlayered with 7 ml of soft APT agar (0.75% agar) that was seeded with a 1% inoculum of the indicator strain. The plates were incubated at different temperatures to allow growth of the indicator strain. Zones of inhibition were measured as the diameter (mm) across a zone of inhibition of growth of the indicator organism (Tagg *et al.*, 1976).

2.2.3. Effect of medium and inoculum size on growth and bacteriocin production by *C. maltaromaticum* UAL26

C. maltaromaticum UAL26 was inoculated from frozen stock into tempered broth (APTNT, 15°C; RPMI, 4°C) and subcultured twice using a 1% inoculum volume. The third subculture was inoculated (APTNT, 1L; RPMI, 2L) using a 0.1% inoculum volume. Samples were removed at hourly or daily intervals. Counts of *C. maltaromaticum*

UAL26 were determined by preparing standard serial dilutions and spread-plating 100 µl samples onto the surface of prepoured plates of APT agar. Counts were determined in duplicate and converted to log CFU/ml and then averaged. The pH of the broth was measured using an Accumet AR15 pH meter (Fisher Scientific). Bacteriocin activity was measured by the spot-on-lawn assay described in section 2.2.2.

C. maltaromaticum UAL26 was subcultured from frozen stock into liquid broth media (APT, APTNT, CAA, CAANT, or RPMI) at 4°C, subcultured twice, and the second subculture was assayed for bacteriocin titre by spot-on-lawn using *L. monocytogenes* ATCC 43256 as the indicator organism. The second subculture of *C. maltaromaticum* UAL26 in APT, APTNT, CAA, and CAANT was subcultured into RPMI medium at an inoculum of 1% vol./vol. to measure the effect of switching from complex media to a minimal medium on bacteriocin production.

To determine the effect of inoculum size on bacteriocin production, the second subculture of *C. maltaromaticum* UAL26 in APT, APTNT, and CAA was serially diluted from 10^{-1} to 10^{-10}, grown at 4°C for 5 d, and tested for bacteriocin production by the spot-on-lawn assay.

2.2.4. Effect of time and temperature on bacteriocin production by *C. maltaromaticum* UAL26

To determine the effect of incubation temperature on bacteriocin production, *C. maltaromaticum* UAL26 was subcultured from frozen stock into APT or APTNT broth media that had been tempered and incubated at temperatures between 1°C and 25°C, until the culture had reached maximum population (1 to 12 d). Two successive subcultures were done, and the third subculture was tested for bacteriocin titre by the spot-on-lawn assay.

To determine the effect of a change in incubation temperature on bacteriocin production, *C. maltaromaticum* UAL26 was subcultured from frozen stock into APT or APTNT broth that had been held at 15°C for at least 1 h before inoculation, incubated at 15°C, and subcultured daily or once every 2 d. During incubation, cultures were assayed by standard spread-plate count to ensure that maximum population had been reached, and for bacteriocin activity by the spot-on-lawn assay. The second subculture that had detectable antibacterial activity was subcultured into APT or APTNT broth at 25°C, and either held at 25°C without further subculturing for up to 5 d, or subcultured up to three more times at 25°C. These cultures were also tested daily for bacteriocin production using a spot-on-lawn assay.

2.3. Results

2.3.1. Antibacterial activity of *C. maltaromaticum* UAL26

Table 2.2 lists the inhibitory activity measured by the deferred inhibition assay of *C. maltaromaticum* UAL26 grown at different temperatures. *C. maltaromaticum* UAL26 was most active against the indicator organisms when both the producer and indicator organisms were grown at 10°C. *C. maltaromaticum* UAL26 had the largest zones of inhibition against indicator strains of *L. monocytogenes*.

2.3.2. Effect of medium and inoculum size on growth and bacteriocin production by *C. maltaromaticum* UAL26

The viable count (CFU/ml) and the change in media pH was monitored in cultures of *C. maltaromaticum* UAL26 grown at 4°C for 70 d in RPMI, a minimal medium

Table 2.2. Antibacterial activity of *C. maltaromaticum* UAL26 grown at 10, 15, and 25°C against indicator strains grown at 10, 15, or 25°C.

	Growth temperature of *C. maltaromaticum* UAL26 (°C)								
	10			15			25		
Indicator organism / growth temperature (°C)	10	15	25	10	15	25	10	15	25
C. maltaromaticum UAL26	–	–	–	–	–	–	–	–	–
C. maltaromaticum LV17	++	++	++	+	–	+	+	+	–
C. divergens LV13	+++	+++	+++	++	++	++	–	+	–
Br. campestris ATCC 43754	+++	+	+++	++	++	++	++	++	++
Br. thermosphacta ATCC 11509	++	++	++	++	++	++	+	+	+
Ent. faecalis ATCC 7080	++	++	++	+	++	+	+	+	+
Ent. faecium ATCC 19434	+++	+++	++	++	++	++	++	++	+
Ent. faecium BFE900	++	++	++	++	++	++	++	++	+
Lc. lactis ssp. *lactis* ATCC 11454	++	++	++	++	++	++	++	++	++
Lb. sakei 706	+++	++	++	++	++	++	++	+	+
Lb. sakei 1218	++	++	++	++	+	+	++	–	+
Lb. sakei 185	++	++	++	+	+	+	+	+	+
Leu. gelidum UAL187	++	++	++	+	++	+	+	+	+
Leu. mesenteroides Y105	++	++	++	+	+	++	–	–	+
P. acidilactici PAC1.0	++	++	++	++	++	++	–	+	++
L. monocytogenes ATCC 15313	++++	+++	+++	+++	++	++	–	+	–
L. monocytogenes CDC 7762	+++++	++++	++++	++++	+++	+++	+	+	+
L. monocytogenes ATCC 43256	++++	++++	++++	++++	+++	+++	–	+	–
L. monocytogenes HPB 642	+++	+++	+++	+++	++	++	–	–	–
L. monocytogenes UAFM 1	+++++	++++	++++	++++	+++	+++	–	–	–
L. monocytogenes UAFM 15	+++++	++++	++++	++++	+++	+++	–	–	–

(--) No inhibition, or small rings of inhibition around producer colony of up to 1 mm width assumed to be due to acid diffusion, (+) inhibition zone up to 1 cm diameter, (++) inhibition zone up to 2 cm diameter, (+++) inhibition zone up to 3 cm diameter, (++++) inhibition zone up to 4 cm diameter, (+++++) inhibition zone of 5 cm diameter or larger.

(Figure 2.1). The population reached a maximum of 10^8 to 10^9 CFU/ml within 14 d of incubation. The pH of the RPMI media dropped from 7 to 4.5 by the end of the experiment. Bacteriocin titre was tested but no activity was detected.

When grown in APTNT at 15°C, the population of *C. maltaromaticum* UAL26 reached a maximum of 10^9 to 10^{10} CFU/ml after 30 h of incubation (Figure 2.2). The pH of the APTNT media dropped from 7 to 5.0 by the end of the experiment. Bacteriocin activity was detected in the APTNT broth in the late logarithmic phase of growth, and reached a final titre of 400 A.U./ml.

The effect of growth medium composition and inoculum size on bacteriocin production showed that *C. maltaromaticum* UAL26 produced the highest titre of bacteriocin when grown in APTNT (Figure 2.3). Growth in APT broth produced less activity than was observed in APTNT, and growth in CAA generated almost negligible amounts of activity. Bacteriocin activity was observed in APTNT at inoculum volumes from 10^{-1} to 10^{-8} CFU/ml, in APT broth from 10^{-1} to 10^{-6} CFU/ml, and in CAA broth from 10^{-2} and 10^{-3} CFU/ml. *C. maltaromaticum* UAL26 did not produce detectable amounts of bacteriocin activity in CAANT or RPMI throughout the experiment (data not shown). Switching from a rich medium (APT or APTNT) to a defined medium (RPMI) had no effect on production of bacteriocin activity (data not shown).

2.3.3. Effect of time and temperature on bacteriocin production by *C. maltaromaticum* UAL26

Growth of *C. maltaromaticum* UAL26 in APTNT broth produced greater antibacterial activity over a broader range of temperatures than growth in APT broth (Figure 2.4). In APTNT, the maximum temperature for production of detectable amounts

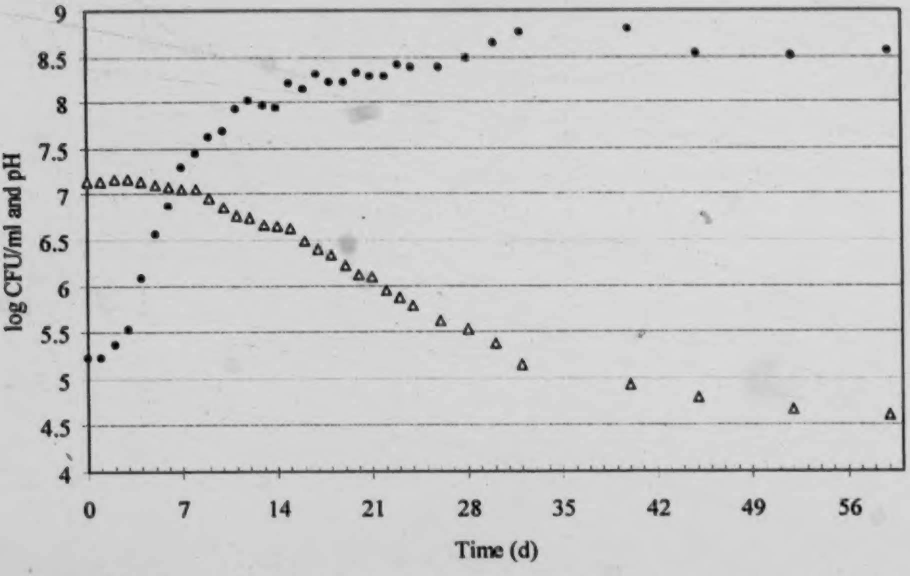

Figure 2.1. Viable count (●) and change in pH (△) of *C. maltaromaticum* UAL26 grown at 4°C in RPMI. (n=2)

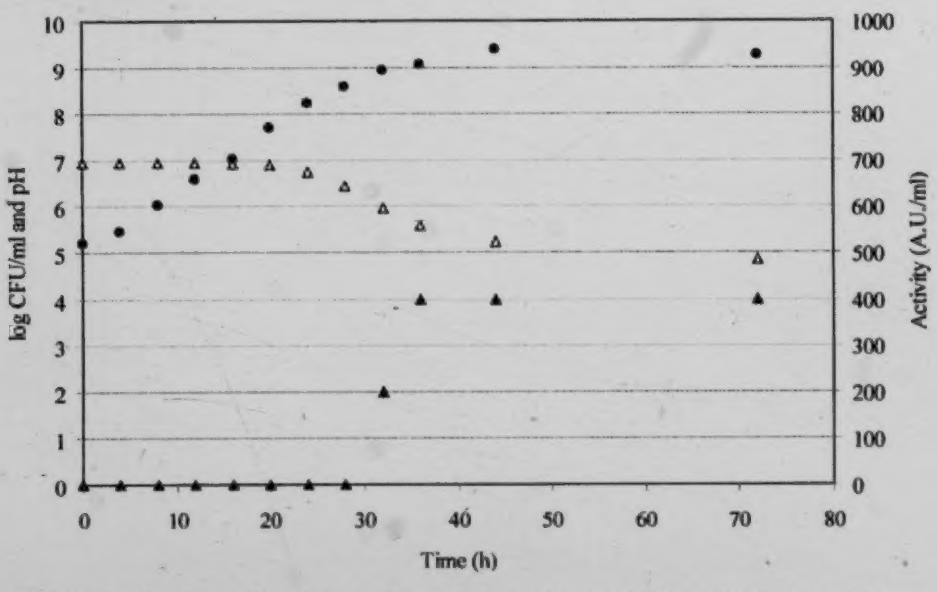

Figure 2.2. Viable count (●), change in pH (△), and bacteriocin activity (▲) of *C. maltaromaticum* UAL26 grown at 15°C in APTNT. *L. monocytogenes* ATCC 43256 was used as the indicator organism. (n=2)

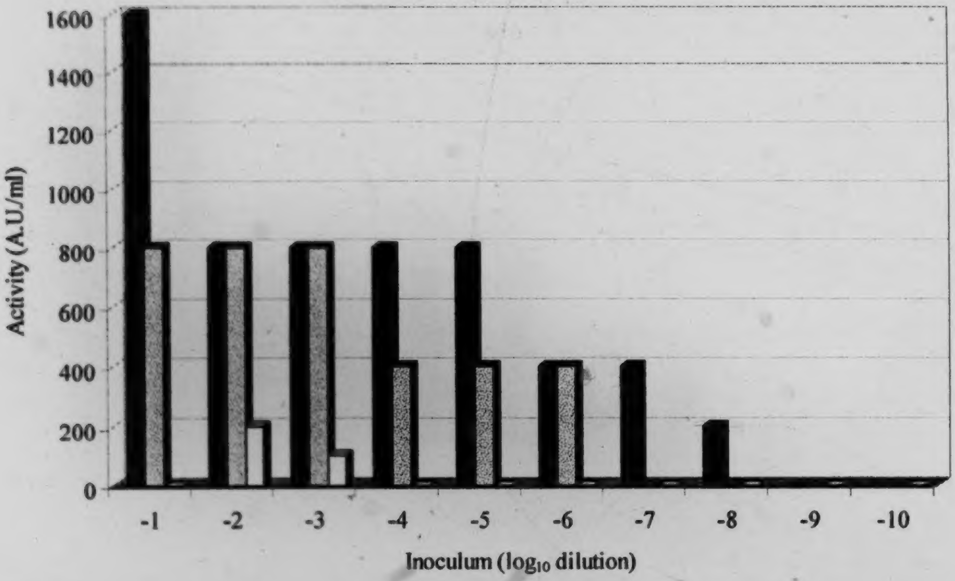

Figure 2.3. Effect of inoculum size on bacteriocin production by *C. maltaromaticum* UAL26 grown in APTNT (■), APT (▨), and CAA (☐) at 4°C for 5 d. *L. monocytogenes* ATCC 43256 was used as the indicator organism. (n=2)

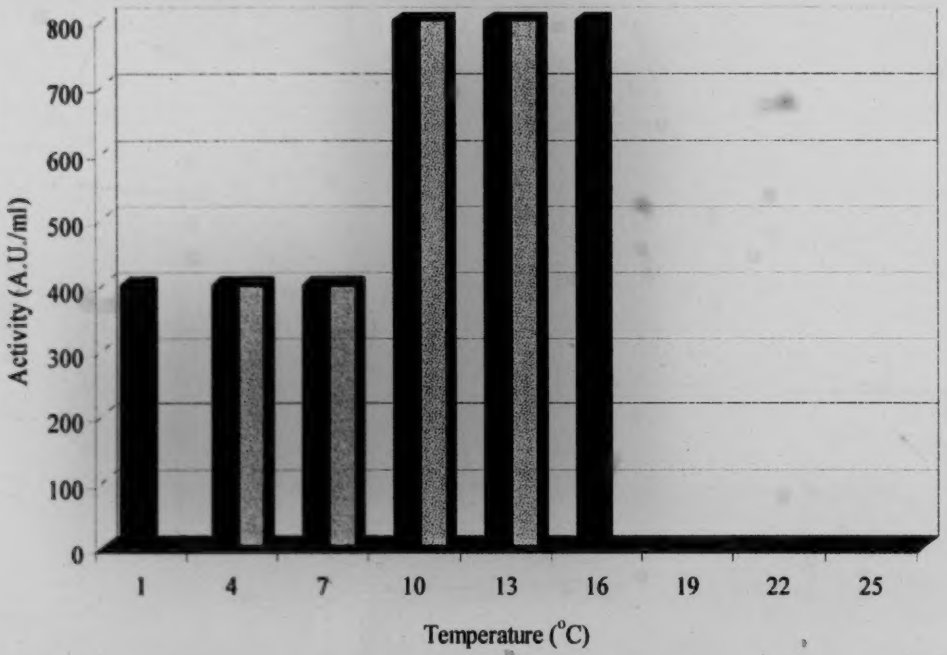

Figure 2.4. Effect of incubation temperature on bacteriocin production by *C. maltaromaticum* UAL26 grown in APTNT (■) and APT (▨) broths. *L. monocytogenes* ATCC 43256 was used as the indicator organism. (n=3)

of bacteriocin was 16°C, and bacteriocin was produced at the lower temperature limit of the assay (1°C). Bacteriocin production in APT was restricted to a range of 4°C to 13°C. At incubation temperatures where bacteriocin activity was detected in broth media, the titre of activity was equal.

The optimum temperature for bacteriocin production in regard to time of fermentation is summarized in Table 2.3. The most efficient growth temperature for bacteriocin production in *C. maltaromaticum* UAL26 is 15°C. Bacteriocin production was detected at this temperature within 3 subcultures from a frozen stock culture. At 15°C, subcultures took 24 h to grow to full population, and these cultures had the highest titre of bacteriocin activity (800 A.U./ml) once adapted to production.

Table 2.3. Effect of growth and subculture time on bacteriocin production by *C. maltaromaticum* UAL26 grown in APTNT broth.

Temperature of growth (°C)	Time (d) for subculture growth	Total time (d) of growth from frozen stock	Bacteriocin activity (A.U./ml)
4	~3	~10	400
10	~2	~6	800
15	~1	~3	800
25	~1	~3	0

The effect of subculturing daily or every second day showed that regardless of frequency of subculture, *C. maltaromaticum* UAL26 in APTNT produced detectable bacteriocin after 3 d, while subcultures in APT only produced bacteriocin after 5 d (Table 2.4). When a culture that was producing antibacterial activity at 15°C was subcultured and incubated at 25°C, the amount of detectable activity rapidly decreased. In a culture held continuously at 25°C without further subculturing, activity remained detectable for 4 d regardless of medium type. With further subculturing under the same conditions, activity was undetectable after 3 d, again regardless of medium type.

Table 2.4. Effect of frequency of subculturing and temperature of incubation on bacteriocin production in *C. maltaromaticum* UAL26 grown at 15°C and moved to 25°C, using a spot-on-lawn assay. *L. monocytogenes* ATCC 43256 was used as the indicator organism.

	Subculture frequency and growth temperature							
	Daily 15°C		Every 2 days 15°C		15°C subcultured into 25°C and held		15°C moved to 25°C subcultured daily	
Day	APT	APTNT	APT	APTNT	APT	APTNT	APT	APTNT
1	--	--	--	--	+	+	+	+
2	--	--			+	+	~	~
3	--	+	--	+	+	+	--	--
4	--	+			~	~	--	--
5	+	+	+	+	--	--		
6	+	+						
7			+	+				
8								
9			+	+				

(+) Inhibition ≥ 100 A.U./ml, (~) Inhibition < 100 A.U./ml, (--) No visible zone of inhibition.

2.4. Discussion

The antibacterial activity produced by *C. maltaromaticum* UAL26 has a spectrum of activity against a variety of gram-positive organisms, with many of the sensitive species being members of the lactic acid bacteria. The spectrum of activity includes food spoilage organisms, such as *Lb. sakei*, and it has strong antibacterial activity against the pathogen *L. monocytogenes*. *C. maltaromaticum* UAL26 produces higher levels of detectable activity against the indicator organisms tested when it is grown at lower temperatures. The activity also increases when these indicators are also grown at lower temperatures, possibly due to greater susceptibility of the indicator organism to inhibition by the antibacterial compound (Buchanan and Klawitter, 1992). The majority of the difference in activity results from temperature of growth of the producer organism, rather than the indicator, as observed by the increase and consistency in the diameter of zones of inhibition in deferred inhibition assays when the producer organism is grown at 10°C as compared to 25°C.

Both complex (APTNT) and minimal (RPMI) media formulations resulted in typical bacterial growth curves. The pH of the media decreased in the expected fashion. Bacteriocin production was not detected in RPMI, but it could be detected in the late logarithmic growth phase in APTNT, and reached a maximum when the culture entered the stationary growth phase, based on the plateau of A.U./ml observed at this point of the growth curve.

The trend of loss of detection of antibacterial activity with decreasing subculture inoculum volume suggests that the value of using larger inoculation volumes is two-fold: an increase in final bacteriocin titre, and a reduction in the time needed for a culture to reach its maximum population density, and thereby maximum bacteriocin activity. However, if the culture has not been adapted to growth at a temperature permissive to bacteriocin production (<19°C), large inoculum size may cause the culture to grow to maximum population too quickly to produce bacteriocin, as shown in Figure 2.3 for CAA medium. The observation that bacteriocin production is not detectable below a certain inoculum level (below 10^{-9} in APTNT, 10^{-6} in APT, and 10^{-3} in CAA) may be due to dilution of an induction peptide to below the concentration needed to induce bacteriocin production, as observed in *C. maltaromaticum* LV17 (Saucier *et al.*, 1995).

A cutoff temperature exists for the detection of bacteriocin activity in the supernatant of *C. maltaromaticum* UAL26. This temperature (>16°C for *C. maltaromaticum* UAL26 grown in APTNT) is well below the optimum growth temperature of the organism. Increases in bacteriocin production when cultures are grown at lower temperatures have been observed for *C. maltaromaticum* (Buchanan and Klawitter, 1992; Pilet *et al.*, 1995; Campos *et al.*, 1997), *C. divergens* (Pilet *et al.*, 1995), *Lactobacillus sakei* (Moretro *et al.*, 2000), and *Lactococcus lactis* ssp. *lactis* (Keren *et al.*, 2004), but none report a specific temperature cutoff point as observed in *C. maltaromaticum* UAL26. Other intrinsic factors have also been reported as having an influence on bacteriocin production. Not only may temperature play a role, but a culture may require a particular pH (Hindre *et al.*, 2004), osmotic concentration (Sashihara *et al.*, 2001), competitor microflora (Maldonado *et al.*, 2004), or proteolytic enzymes (Gomez *et al.*, 2002) to stimulate bacteriocin production.

Bacteriocin production by *C. maltaromaticum* UAL26 was originally shown to be affected by the temperature of incubation during growth on solid media. This was evaluated against a pair of indicator organisms using the deferred inhibition method of measuring antibacterial antagonism (Burns, 1987), and later against four more indicator organisms (Gursky *et al.*, 2002). The results of the current study showed that not only does temperature play a role, but also medium type and the components of the medium. Production of increased titres of bacteriocin by *C. maltaromaticum* UAL26 occurs at temperatures below 16°C, and only in complex media such as APT or CAA, but not in a minimal medium such as RPMI. In CAA, Tween 80 (a surfactant) increased the amount of the bacteriocin from undetectable to 200 A.U./ml. However, the inclusion of Tween 80 in APT resulted in no activity at the high (16°C) and low (1°C) temperatures of incubation. This finding is different from the effect of Tween 80 on bacteriocin production observed in production of lacticin RM by *Lc. lactis* ssp. *lactis* (Keren *et al.*, 2004), which demonstrated that Tween 80 increased the concentration of bacteriocin eightfold in tryptic soy yeast broth. There are several other examples in the literature that show that Tween 80 improves production of bacteriocin (Huot *et al.*, 1990; Parente and Hill, 1992; Moretro *et al.*, 2000). Tween 80 may act by preventing the aggregation of bacteriocin molecules (Huot *et al.*, 1990; Nissen-Meyer *et al.*, 1992); however, the effect of Tween 80 on bacteriocin producing cultures, and on bacteriocins in broth culture, may differ for each bacteriocin. In the current study, it is possible that other differences in media composition between APT (a standardized medium) and APTNT (formulated from individual components of APT) could contribute to the variation in bacteriocin production.

C. maltaromaticum UAL26 should be grown for the purpose of bacteriocin production within the temperature that permits production of detectable levels of bacteriocin in the supernatant (1 to 19°C). To optimize the time needed to prepare a large volume of culture for bacteriocin isolation, growth at 15°C in APTNT reached maximum population in 24 h, produced the highest titre of bacteriocin in the supernatant, and was sufficiently below the temperature cutoff of 19°C to ensure that any variation in incubator temperature would not affect bacteriocin production.

Growth in APTNT allows faster adaptation to increased bacteriocin production by *C. maltaromaticum* UAL26. Regardless of subculture frequency, cultures grown in APTNT produced bacteriocin in the supernatant after 3 d of incubation at 15°C. Growth in APT was more restrictive, as it allowed the full titre of bacteriocin to be produced after 5 d of incubation at 15°C. It took 3 d for the metabolic processes of *C. maltaromaticum* UAL26 to fully adapt to cold incubation temperatures (i.e. 15°C), but increased bacteriocin production is rapidly lost when a reduced growth temperature-adapted culture is subcultured and incubated at 25°C. The loss of bacteriocin production when cultures were moved from 15°C to 25°C suggests that bacteriocin production in *C. maltaromaticum* UAL26 is dependent on a mechanism of adaptation to change in incubation temperature. Temperatures that permit high levels of bacteriocin production are sub-optimal for the growth rate of *C. maltaromaticum* UAL26. *C. maltaromaticum* UAL26 subcultured at a 1% inoculum reached maximum population within 16 h when grown at 25°C, but took 24 h when grown at 15°C.

Amino acid sequencing of a bacteriocin by Edman degradation requires that enough active peptide is present in the supernatant so that purification will result in a quantity of pure peptide that can be sequenced. Previous attempts to sequence the bacteriocin from *C. maltaromaticum* UAL26 failed because an insufficient quantity of peptide was produced to allow for successful purification. In purifications where a desired biological compound is present in too low of a concentration to be isolated, two solutions are typically applied: increase the culture volume, or alter the fermentation conditions to increase production of the desired compound. At some point, increase of culture volume becomes prohibitive in regard to time and labour required for purification. Therefore, the conditions of culture growth or metabolism must be altered in such a way to increase production levels. Discovery of the temperature dependent nature of bacteriocin production in *C. maltaromaticum* UAL26 allows further research on purification and characterization of the bacteriocins produced by this organism.

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3. PURIFICATION AND IDENTIFICATION OF A BACTERIOCIN PRODUCED BY *Carnobacterium maltaromaticum* UAL26, AND GENETIC CHARACTERIZATION OF BACTERIOCINS PRODUCED BY *C. maltaromaticum* UAL26 AND *C. maltaromaticum* JG126

3.1. Introduction

Purification of bacteriocins can be challenging because of the low concentrations that may be present in culture supernatant, or because of the chemical nature of the bacteriocin. As bacteriocins often contain hydrophobic regions and carry ionic charge, chromatography methods which separate compounds based upon hydrophobicity and charge are useful for purification. Hydrophobic interaction (Queiroz *et al.*, 2001), ionic exchange (Choudhary and Horvath, 1996), and molecular size exclusion (Irvine, 2003) are three of the most common principles used in column chromatography purifications. These methods are often used in combination in a series of purifications. Other methods, such as ammonium sulphate precipitation (Hastings *et al.*, 1991; Holck *et al.*, 1996; Herbin *et al.*, 1997; Schobitz *et al.*, 1999), solvent extraction (Burianek and Yousef, 2000), detergent phase partitioning (Metivier *et al.*, 2000), affinity column (Gibbs *et al.*, 2004), and immunoaffinity purification (Suarez *et al.*, 1997) have also been demonstrated as useful methods to purify bacteriocins, although some of these methods require the bacteriocin to have been previously purified and characterized. Continuous fermentation has also been demonstrated as a method to increase bacteriocin production (Parente and Ricciardi, 1999).

Several bacteriocins have been characterized from *Carnobacterium* spp., including carnobacteriocin A (Worobo *et al.*, 1995), carnobacteriocin BM1 (Quadri *et al.*, 1994), carnobacteriocin B2 (Quadri *et al.*, 1994), piscicolin 126 (Jack *et al.*, 1996), carnocin UI49 (Stoffels *et al.*, 1992), carnocin H (Blom *et al.*, 2001), divergicin A (Worobo *et al.*, 1995), divergicin 750 (Holck *et al.*, 1996), and divercin V41 (Metivier *et al.*, 1998). These bacteriocins were purified using hydrophobic interaction, ionic exchange, or size exclusion chromatography, or a combination of these, and other methods.

The difficulty in detecting bacteriocin activity in culture supernatant of *C. maltaromaticum* UAL26 prevented purification of its bacteriocin activity (Rosario, 2001). The bacteriocin was known to be proteinaceous, as studied by proteolysis sensitivity in deferred inhibition assays of *C. maltaromaticum* UAL26 (Burns, 1987). Other characteristics of the bacteriocin, such as heat and pH stability, or the phase of culture growth during which bacteriocin is produced, could not be evaluated because of the inability to detect the bacteriocin in the culture supernatant. The potential for isolation of sufficient quantities of peptide for sequencing and identification increased with the discovery that reduction in growth temperature increased bacteriocin production, which allowed detection of antibacterial activity in broth media (Chapter 2, this thesis).

The possibility of isolating enough bacteriocin for sequencing led to the goal of using reverse genetics to identify and sequence the structural gene encoding the bacteriocin produced by *C. maltaromaticum* UAL26. Examination of the bacteriocin operon, including its methods of immunity, induction, regulation, and transport (secretion into the surrounding media), by genetic characterization could then be done. The primary objectives of this research were to purify and characterize the bacteriocins produced by *C. maltaromaticum* UAL26 and to characterize the genetic elements involved in bacteriocin production.

3.2. Materials and methods

3.2.1. Bacterial strains and growth media

C. maltaromaticum UAL26 was acquired from the University of Alberta Lactic Acid Bacteria collection (University of Alberta, Edmonton, AB). *C. maltaromaticum* JG126 was acquired from the Australian Food Industry Science Centre (Werribee, Victoria, Australia). Stock cultures of *C. maltaromaticum* UAL26 and *C. maltaromaticum* JG126 were maintained at -70°C in All Purpose Tween broth (APT; Difco; BD Diagnostics, Sparks, MD) with glycerol (20% vol./vol.; Fisher Scientific, Edmonton, AB). Cultures of *C. maltaromaticum* UAL26 and JG126 used in this study were prepared by inoculation of cells from frozen stock culture into tempered broth (by holding the media at 15 or 25°C for at least 1 h before inoculation). Cultures were subcultured twice more at an inoculation volume of 1% vol./vol. before use in experiments. *C. maltaromaticum* UAL26 was grown at 15°C in APT without Tween 80 (APTNT), made using the formulation of the manufacturer, except for the exclusion of the Tween 80. Stock cultures of *C. maltaromaticum* UAL26 adapted to production of bacteriocin at 15°C were maintained at -70°C in APTNT with glycerol (20% vol./vol.).

3.2.2. Antibacterial activity of *C. maltaromaticum* UAL26

Listeria monocytogenes ATCC 43256 was grown from frozen stock culture in APT broth at 37°C for 24 h, and subcultured at least once before use in experiments as the indicator organism to determine arbitrary activity units (A.U.) of bacteriocin activity in spot-on-lawn assays. Arbitrary activity units were calculated as the reciprocal of the highest dilution in a doubling dilution series that showed a clear zone of inhibition, and are expressed in A.U./ml (Ahn and Stiles, 1990). For the spot-on-lawn assay, 10 μl of each dilution of cell-free (centrifuged at 10,000 x *g* for 5 min), heated (65°C for 30 min) supernatant was spotted onto APT agar plates, and overlayered with 7 ml of soft APT agar (0.75% agar) seeded with a 1% inoculum of *L. monocytogenes* ATCC 43256. Plates were incubated at 37°C for 24 h, and zones of inhibition were measured and A.U./ml calculated.

3.2.3. Protease sensitivity, temperature, and pH stability of a bacteriocin from *C. maltaromaticum* UAL26

C. maltaromaticum UAL26 was grown for three subcultures in 100 ml of APTNT broth at 15°C. Preparations of cell-free supernatant containing bacteriocin activity were made by centrifugation of 1 ml of fully grown culture (12,000 x *g* for 5 min) in a 1.5 ml microcentrifuge tube, pipetting the supernatant into a new microcentrifuge tube, and heating at 65°C for 30 min. Sensitivity of the bacteriocin produced by *C. maltaromaticum* UAL26 to proteolytic enzymes was evaluated using the following modified method of the spot-on-lawn assay. Proteinase K (Sigma-Aldrich Canada, Oakville, ON), pronase E (Sigma-Aldrich), and trypsin (Sigma-Aldrich) were stored as 1 mg/ml stock solutions at -70°C. The active supernatant (10 μl) was spotted onto an APT agar plate and allowed to dry. Once dried, 10 μl of a 1 mg/ml solution of protease was spotted 1 cm away from the first spot and allowed to dry before the plate was overlayered with 7 ml of soft APT agar inoculated with 1% vol./vol. of a culture of *L. monocytogenes* ATCC 43256.

Stability of the bacteriocin across a range of temperatures was evaluated by exposing 1 ml samples of cell-free supernatant of *C. maltaromaticum* UAL26 grown at 15°C to temperatures ranging from -80°C to 121°C. These samples were assayed for bacteriocin activity at various time intervals by the spot-on-lawn assay.

Stability of the bacteriocin across a pH range from 0.5 to 12 was evaluated by altering the pH of 1 ml samples of cell-free, heated supernatant of *C. maltaromaticum* UAL26 containing bacteriocin activity using either hydrochloric acid (HCl; Fisher Scientific) or sodium hydroxide (NaOH; Fisher Scientific). These samples were stored at 4°C for up to 40 d, and tested for activity at various time intervals using a spot-on-lawn assay.

3.2.4. Purification of a bacteriocin from *C. maltaromaticum* UAL26

Prior to sterilization of the APTNT media to be used for fermentation, a 1 L volume of the medium without glucose was prepurified by washing it through an Amberlite XAD-16 resin column (Sigma-Aldrich). The medium was sterilized and the glucose was added by filter sterilization (10 µm low-protein binding PVDF Millex-GV Durapore syringe filter; Millipore, Cork, Ireland). *C. maltaromaticum* UAL26 was grown from frozen stock and subcultured at 1% inoculum three times at 15°C in prepurified APTNT broth. The culture was inoculated (1% vol./vol.) into 1 L of prepurified APTNT broth. After the culture had grown for 24 h it was centrifuged at 16,000 x *g* at 10°C for 15 min, and the cell-free supernatant was subjected to a series of column purifications.

The first column (2.5 x 30 cm) was prepared by shaking a slurry of ~50 to 70 g of Amberlite XAD-16 nonionic polymeric adsorbent resin (Sigma-Aldrich) in 300 ml Milli-

Q water for 10 min, then pouring the column by gravity flow-through. Milli-Q water (300 ml) was used to pack and rinse the column before the centrifuged culture supernatant was loaded onto the column. Once the supernatant was loaded onto the column by gravity flow-through, 500 ml of 30% ethanol (Fisher Scientific) solution was rinsed through the column by pumping (Econo Pump; BioRad, Hercules, CA) at a constant rate of 20 ml/min, followed by 500 ml of 70% propan-2-ol (Fisher Scientific) adjusted to pH 2 by addition of 1 M HCl at 20 ml/min to elute the bound bacteriocin. This fraction was concentrated by rotary evaporation (Rotavapor R-205, vacuum V-511, controller V-805, heating bath B-490; Büchi Labortechnik, Flawil, Switzerland) under vacuum of approximately 34 mbar with gentle heating at 30°C to remove the solvent and to decrease the working volume to approximately 100 ml.

The second column (2.5 x 50 cm) was prepared by shaking a slurry of ~150 to 200 g of Amberlite IR-120 PLUS strongly acidic cationic exchanger resin (Sigma-Aldrich) in 400 ml of 75% propan-2-ol for 15 min. The column was poured by gravity flow-through, and 500 ml of 75% propan-2-ol was used to pack and rinse the column. Milli-Q water (1 L) was rinsed through the column by gravity flow-through to restore the hydration of the resin. The 100 ml concentrate from the Amberlite XAD-16 column purification was applied by gravity flow-through, and washed successively with the following solutions: 500 ml Milli-Q water by gravity flow-through, 500 ml 1 M NaCl by gravity flow-through, and 500 ml 50% propan-2-ol by pump (20 ml/min). The bacteriocin was eluted from the cation-exchange column with 500 ml 50% propan-2-ol containing 0.1 M NaCl at 20 ml/min. This fraction was concentrated by rotary evaporation under vacuum of approximately 34 mbar with gentle heating at 30°C to remove the solvent and decrease the working volume to approximately 50 ml.

The third column was a C_{18} Mega-Bond Elut (10 g, 60 ml) disposable cartridge (Varian Canada, Inc., Mississauga, ON), prepared for use by vacuum filtering 1 column volume (~60 ml) of 100% methanol (Fisher Scientific) through the column, followed by 1 column volume of Milli-Q water. The 50 ml concentrate from the Amberlite IR-120 PLUS cation exchange column purification was applied by vacuum filtering, and washed successively with 1 column volume of each of the following solutions: Milli-Q water, 30% ethanol, 30% acetonitrile (Fisher Scientific), 20% propan-2-ol, 40% propan-2-ol. The bacteriocin was eluted with 40 ml of 70% propan-2-ol adjusted to pH 2. This fraction was concentrated by rotary evaporation under vacuum of approximately 34 mbar with gentle heating at 30°C to remove the solvent and decrease the working volume to approximately 5 ml.

The final purification was done on a HPLC apparatus (System Gold with programmable solvent module 126 pumps using 32 Karat software; Beckman Coulter, Fullerton, CA), using a C_{18} Protein and Peptide Semi-preparative RP-HPLC column (model #218TP510 with a 259FSK810 guard column; Grace-Vydac, Hesperia, CA). Separation was done using a 0.1% trifluoroacetic acid (TFA; Sigma-Aldrich) in Milli-Q water (solvent A):propan-2-ol with 0.1% TFA (solvent B) gradient of the following method: a gradient from 25 to 35% solvent B for 15 min, a gradient from 35 to 80% solvent B for 0.5 min, 80% solvent B for 5 min, a gradient from 80 to 20% solvent B for 0.5 min, and 20% solvent B for 9 min. Fractions were collected based on polypeptide absorbance at 220 nm (System Gold 166 detector; Beckman Coulter). Bacteriocin activity was determined for each fraction using a spot-on-lawn assay. The active fractions from 6 runs of HPLC purification were combined and repurified using the HPLC method described above.

3.2.5. MALDI-TOF MS of a bacteriocin from *C. maltaromaticum* UAL26

Samples for MALDI-TOF mass analysis were prepared using 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) as a matrix. Solutions containing the sample peptide were mixed in a 1:1 ratio (vol./vol.) with a stock solution of sinapinic acid (10 mg/ml) in 60% acetonitrile containing 0.1% TFA before depositing them on the probe. To prepare the samples on the probe, sinapinic acid (0.7 µl; 4 mg/ml) in a 1:1 acetone:methanol mixture was pipetted onto a gold target plate. The solvent was allowed to evaporate, leaving a thin layer of sinapinic acid on the surface of the probe. Sample:matrix solution (0.3 µl) was dried on top of this layer at a pressure of 1 atm. Mass spectra were recorded using a single-stage reflectron MALDI-TOF mass spectrometer (API QSTAR Pulsar with an oMALDI source; Applied BioSystems, Foster City, CA).

3.2.6. Amino acid sequencing of a semi-purified bacteriocin from *C. maltaromaticum* UAL26

A portion of the semi-purified bacteriocin sample was sent to the Alberta Peptide Institute (University of Alberta) for amino acid sequencing. Amino acid sequencing was done using a HP1005A protein sequencer running Routine 3.1 for HPLC detection of phenylthiohydantoin-amino acids (PTH-a.a.'s). The amino acid sequencer was coupled to a HPLC instrument (Series II 1090; Hewlett Packard, Palo Alto, CA). Automated Edman degradation of the peptide sample generated isothiocyanate derivatives and TFA-catalyzed cleavage of amino acids. Downstream HPLC characterization of PTH-a.a.'s was achieved using a C_{18} analytical HPLC column (Hypersil C_{18} 2.1 x 250 mm 5 μm; Agilent, Palo Alto, CA). The HPLC method used a two-solvent method: solvent A was 15% acetonitrile containing 0.1% TFA, and solvent B was 31% isopropanol containing 0.1% TFA. PTH-a.a.'s were eluted in a gradient from 100% solvent A to 100% solvent B over 16 min at a flow rate of 300 μl/min. The resulting sequence was analysed for similarity to known peptides using a BLAST-protein search (Altschul *et al.*, 1990; NCBI-BLAST, 2004) and by visual examination of the results.

3.2.7. Genetic probing of the piscicolin 126 operon in *C. maltaromaticum* UAL26

DNA primers were designed based on the sequence of piscicolin 126 from *C. maltaromaticum* JG126 (Jack *et al.*, 1996), synthesized (Applied Biosystems 394/8 DNA/RNA Synthesizer using phosphoramidite chemistry; DNA Core Facility, University of Alberta), and used in a PCR reaction (Kleppe *et al.*, 1971; Saiki *et al.*, 1985; Mullis *et al.*, 1986) to amplify the structural gene. To increase the chance of at least one of the possible four primer pairs amplifying the desired region, two primers were selected at the ends of the coding region for the structural gene, and the other two were selected further upstream or downstream of the structural gene. The sequence of the primers was as follows:

primer F1 5'-ATATGAATTCCGATGTTACAATCAATTAAC-3',
primer F2 5'-ATATGAATTCATGAAAACTGTTAAAGAACT-3',
primer R1 5'-ATATTCTAGACTTTTCCTCCAGAAAACCA-3', and
primer R2 5'-ATATTCTAGATTATCCTTTGTTCCAACC-3'.

For all four reactions, DNA was amplified in 100 µl PCR reactions using 0.5 µl of Platinum Taq DNA polymerase High Fidelity (Invitrogen Canada Inc., Burlington, ON), 10 µl of HIFI Platinum Taq Buffer, 4 µl of 50 mM $MgSO_4$, 1 µl of each primer at 200 nM (pairings of the primers listed above), 2 µl of 10 mM dNTP solutions (Invitrogen), and 1 µl of DNA template isolated from *C. maltaromaticum* UAL26. *C. maltaromaticum* UAL26 DNA template was prepared using the following protocol: cell culture (1 ml) was centrifuged (model 5417C; Eppendorf, Hamburg, Germany) at 20,000 x *g* for 5 min, the supernatant was discarded, and the cell pellet was resuspended in 500 µl of sterile Milli-Q water. The cells were centrifuged again, resuspended in 100 µl sterile Milli-Q water, and heated at 100°C for 5 min.

The following PCR protocol was used on a GeneAmp PCR system 2400 thermal cycler (version 2.11; Perkin Elmer Corp., Norwalk, CT): 2 min denaturation at 94°C, 25 cycles of: 1 min denaturation at 94°C, 1 min annealing at 50°C, 1 min extension at 68°C, then a final 5 min extension at 72°C. The samples were cooled and held at 4°C. Each reaction mixture (20 µl) was loaded onto a 1% w./vol. agarose (BD Diagnostics) gel [dissolved in 1 x TBE buffer: 10.778 g of tris(hydroxymethyl)aminomethane (TRIS) (Fisher Scientific), 0.744 g of disodium ethylenediaminetetraacetic acid (EDTA) (Fisher Scientific), 5.5 g of boric acid (Fisher Scientific)/L] and separated in 1 x TBE buffer by electrophoresis at 110 V for approximately 30 min using a Mini-Sub Cell GT gel box (BioRad). The gel was visualized with a Gel Doc 1000 UV transilluminator (BioRad) and photographed.

3.2.8. Identification of the piscicolin 126 operon and sequencing of the structural gene, promoter region, and induction gene in *C. maltaromaticum* UAL26

To determine if the piscicolin 126 operon was present in *C. maltaromaticum* UAL26, primers were designed spanning the piscicolin 126 operon sequence, making use of known conserved amino acid residues or motifs within the genes to be amplified (Axelsson and Holck, 1995; Diep *et al.*, 1996; Dutta *et al.*, 1999; Holland and Blight, 1999; O'Keeffe *et al.*, 1999; O'Connell-Motherway *et al.*, 2000; Kim and Forst, 2001; Fimland *et al.*, 2002; Stephenson and Hoch, 2002; Varughese, 2002), to increase the chance of annealing to *C. maltaromaticum* UAL26 genomic DNA. The primers were synthesized (DNA Core Facility, University of Alberta) and used in paired combinations using the PCR protocol described in section 3.2.7 to probe for the presence of each of the genes in the operon of piscicolin 126. The sequence of the primers was as follows:

primer U2 5'-ATATGAGCTCGTTATTAGTGAATTGACAGAA-3',

primer FI 5'-CTAATATCCATATCTAATATTG-3',

primer RI 5'-ATGGGTAAGTTAAAATGGTTT-3',

primer D1 5'-ATATGGATCCTACAGTACAACCATTTTTATTA-3',

primer FP 5'-AAGTTCTTTAACAGTTTTCAT-3',

primer D2B 5'-ATATCCCGGGTTAACAGTGATAGTATGCCA-3',

primer FN 5'-ATGAACGATAAAAAATACTTG-3',

primer RN 5'-TTAGCATTTTTTAAAAAAAGAAA-3',

primer FK1 5'-TTGTAAAGTATAGTTCTATTA-3',

primer D2A 5'-ATATCCCGGGTTGTTCCAACTGATCAGTG-3',

primer FK2 5'-GATAATGCAATTGAAGCGG-3',

primer RK2 5'-CAATCCTAATCCTTTATGG-3',

primer FR 5'-GAAGATCAAATCATTCAAC-3',

primer RR 5'-TTTGAACTAACCTTTGCCG-3',

primer FT1 5'-GGTGTTAGTGGATCTGGA-3',

primer RT1 5'-CTTCAATAAAGCACGAGCA-3',

primer FT2 5'-GCGACCATGGACTGGAT-3',

primer RT2 5'-ATCCAGTCCACTGGTCGC-3', and

primer RE 5'-AGTGGTACTTGTAGTTTAT-3'.

Amplified DNA fragments and primers surrounding the piscicolin 126 structural gene (F1 and R2 primers), promoter region, and induction gene (FP and RN primers) were sent to the Core DNA Facility (University of Alberta) where the DNA was sequenced by automated capillary electrophoresis (CEQ2000XL DNA Analysis System; Beckman Coulter). DNA sequencing was done using the Sanger dideoxynucleotide termination sequencing protocol. Sequencing reactions were prepared by thermocycler amplification, separated by electrophoresis, and analysed as described in section 3.2.7.

3.2.9. Identification of the carnobacteriocin BM1 structural gene in *C. maltaromaticum* UAL26 and JG126

The carnobacteriocin BM1 gene was originally amplified in *C. maltaromaticum* UAL26 independently of this thesis research (Stiles, unpublished data). To determine if the carnobacteriocin BM1 structural gene was present in *C. maltaromaticum* JG126, DNA primers specific for the known DNA sequence of carnobacteriocin BM1 from *C. maltaromaticum* LV17 were designed by Dr. Marco van Belkum (Canbiocin; University of Alberta, Edmonton, AB) and originally used in other thesis research (Garneau, 2003). Sequences of the primers (DNA Core Facility, University of Alberta) were as follows: primer BM1F1 5'- ATATGAATTCATGAAAAGCGTTAAAGAACT-3', and primer BM1R2 5'-ATATTCTAGATTAAAACCCTGACCAAGC-3'. The piscicolin 126 gene was amplified using primers F1 and R2 described in section 3.2.7.

DNA was amplified in 100 µl PCR reactions using the reagents, enzymes, and DNA template prepared from *C. maltaromaticum* UAL26 and *C. maltaromaticum* JG126 as described in section 3.2.7.

The following PCR protocol was used on a GeneAmp PCR system 2400 thermal cycler (Perkin Elmer): hot start addition of Taq polymerase (D'Aquila *et al.*, 1991; Erlich *et al.*, 1991; Mullis, 1991), 2 min denaturation at 94°C, 35 cycles of: 1 min denaturation at 94°C, 1 min annealing at 50°C, 1 min extension at 68°C, then a final 5 min extension at 72°C, and the samples were cooled and held at 4°C. Each reaction mixture (20 µl) was loaded onto a 1% w./vol. agarose, separated by electrophoresis, and visualized as described in section 3.2.7.

3.3. Results

3.3.1. Protease sensitivity, temperature, and pH stability of a bacteriocin from *C. maltaromaticum* UAL26

The protease sensitivity, and the temperature and pH stability of the bacteriocin activity in cell-free supernatant of *C. maltaromaticum* UAL26 is shown in Table 3.1. The antibacterial activity in the supernatant was sensitive to the three proteolytic enzymes used, which indicates that the antibacterial activity is due to a proteinaceous compound.

Table 3.1. Protease sensitivity, and temperature and pH stability of bacteriocin activity in cell-free supernatant of a culture of *C. maltaromaticum* UAL26 grown at 15°C.

Characteristic		Sensitivity / stability
Protease sensitivity	Proteinase K	+
	Pronase E	+
	Trypsin	+
Temperature stability	121°C	1 h
	100°C	8 h
	65°C	10 h
	≤25°C	>30 d
pH stability	pH 0.5	35 d
	pH 1 to 8	>40 d
	pH 9	21 d
	pH 10	7 d
	pH 11	2 d
	pH 12	<1 d

(+) Sensitive to protease degradation.

The antibacterial activity was very heat stable, withstanding treatment of 121°C for 1 h, and heating at 100°C for 8 h. The bacteriocin activity is also very stable at acidic pH. At alkaline pH, bacteriocin activity begins to decrease. The higher the pH, the more rapid the inactivation; at pH 12, antibacterial activity was not detectable after 24 h.

3.3.2. Purification of a bacteriocin from *C. maltaromaticum* UAL26

The antibacterial activity of the fractions collected after elution from the XAD-16, IR-120 PLUS and C_{18} Mega-Bond columns is summarized in Table 3.2. The sample was eluted from the C_{18} column and purified by RP-HPLC. Antibacterial activity was eluted at approximately 17.5 min. The chromatogram of the pool of fractions collected from the RP-HPLC is shown in Figure 3.1.

Table 3.2. Antibacterial activity detected using the spot-on-lawn assay on samples taken during purification of the supernatant of *C. maltaromaticum* UAL26 grown at 15°C in APTNT using XAD-16, IR120 PLUS, and C_{18} Mega-Bond columns. *L. monocytogenes* ATCC 43256 was used as the indicator organism. (n=4)

Column	Eluent	Bacteriocin activity
XAD-16	30% Ethanol	--
	70% Propan-2-ol pH 2	+
IR120 PLUS	Water	--
	1M Sodium chloride	--
	50% Propan-2-ol	--
	50% Propan-2-ol + 0.1M Sodium chloride	+
C_{18} Mega-Bond	Water	--
	30% Ethanol	--
	30% Acetonitrile	--
	20% Propan-2-ol	--
	40% Propan-2-ol	--
	70% Propan-2-ol pH 2	+

(--) no activity detected, (+) visible zone of inhibition

3.3.3. MALDI-TOF MS of a bacteriocin from *C. maltaromaticum* UAL26

Analysis of MALDI-TOF mass spectrometry data showed a major peak at an experimental mass of 4415.5 Da (Figure 3.2). This molecular mass corresponds with the molecular mass of piscicolin 126, a bacteriocin isolated from *C. maltaromaticum* JG126 (Jack *et al.*, 1996).

3.3.4. Amino acid sequencing of a semi-purified bacteriocin from *C. maltaromaticum* UAL26

Amino acid sequencing of the semi-purified peptide revealed a major sequence of approximately nine residues that was characterised by large PTH-a.a. peak changes between sequencing cycles. The major sequence was composed of the following amino acids, using the common 'single-letter' abbreviation format: H_2N-FARLMASIG-COOH. Smaller PTH-a.a. peak changes were observed in an underlying sequence twelve residues long: H_2N-YYGNGVSCNKNG-COOH. This underlying sequence shared 100% identity

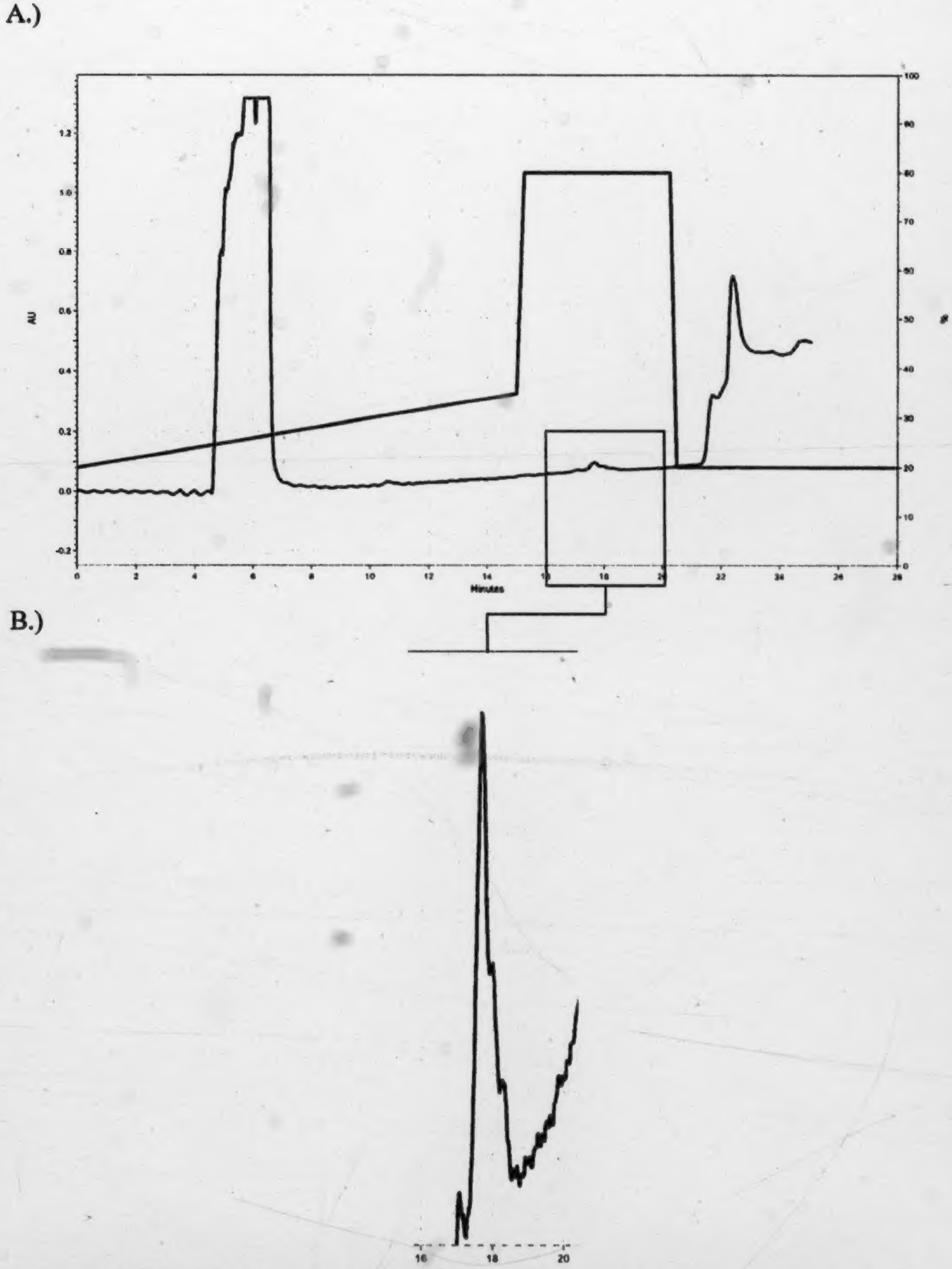

Figure 3.1. RP-HPLC chromatogram of pooled semi-purified bacteriocin from *C. maltaromaticum* UAL26. A.) Detector plot of A_{220nm} and solvent gradient method. B.) Expansion of the peak where bacteriocin activity was detected.

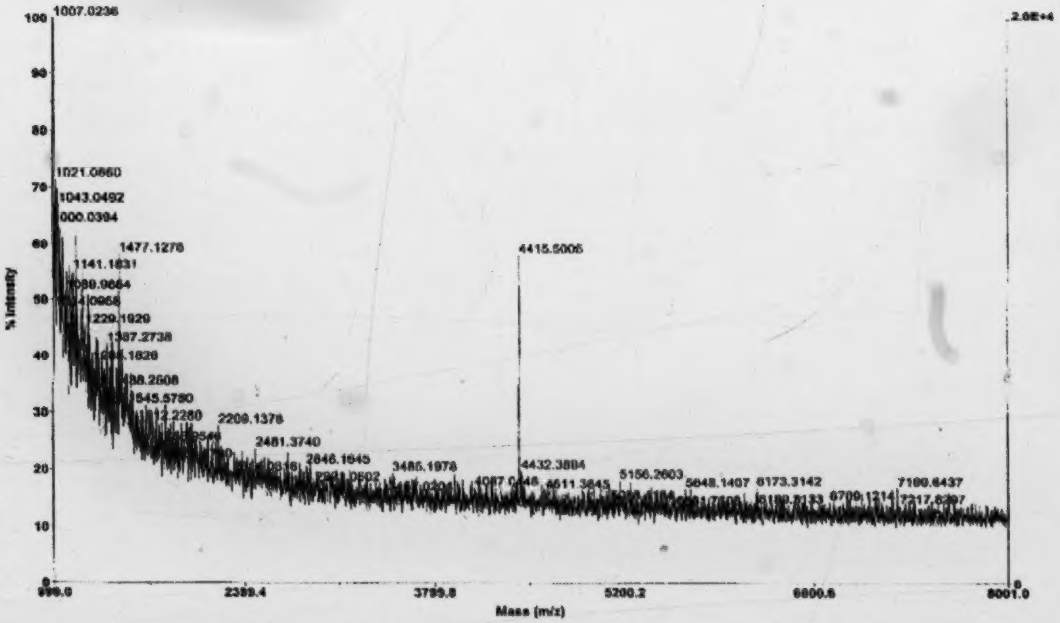

Figure 3.2. MALDI-TOF mass spectrum of C_{18} RP-HPLC semi-purified peptide from *C. maltaromaticum* UAL26.

with the first twelve residues of piscicolin 126 from *C. maltaromaticum* JG126 (Jack *et al.*, 1996) when entered into a BLAST-protein search (Altschul *et al.*, 1990; NCBI-BLAST, 2004) of known bacterial protein sequences. The major peptide sequence was not similar to any known bacteriocins, and the closest protein sequence match was somewhat similar to the neuropeptide CP2 precursor from the eukaryote *Aplysia californica* (California sea hare) (Altschul *et al.*, 1990; NCBI-BLAST, 2004).

3.3.5. Genetic probing of the piscicolin 126 operon in *C. maltaromaticum* UAL26

Primer pairs were chosen surrounding the piscicolin 126 structural gene based on the sequence from the piscicolin 126 operon in *C. maltaromaticum* JG126 (Gibbs *et al.*, 2000). The four sites were chosen to increase the chance that one of the four possible primer pair combinations would amplify the region, given that genetic differences could interfere with the attempt to amplify the gene by PCR. The location of the four primers on either side of the piscicolin structural gene is shown in Figure 3.3, and the expected and observed sizes of each possible combination are shown in Table 3.3. Amplification of fragments by PCR was successful for three of four possible primer combinations, with bands of the expected size detected (Figure 3.4).

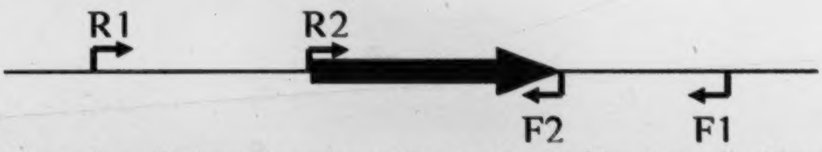

Figure 3.3. Location of primers based upon the sequence of the piscicolin 126 structural gene from *C. maltaromaticum* JG126, and used to probe genomic DNA of *C. maltaromaticum* UAL26.

Table 3.3. Expected and observed sizes of PCR products using primer combinations to amplify the piscicolin 126 structural gene in *C. maltaromaticum* UAL26.

Primer pair	Expected size (bp)	Observed size (bp)
F1 & R1	599	N/A
F2 & R1	463	~450
F1 & R2	324	~350
F2 & R2	188	~200

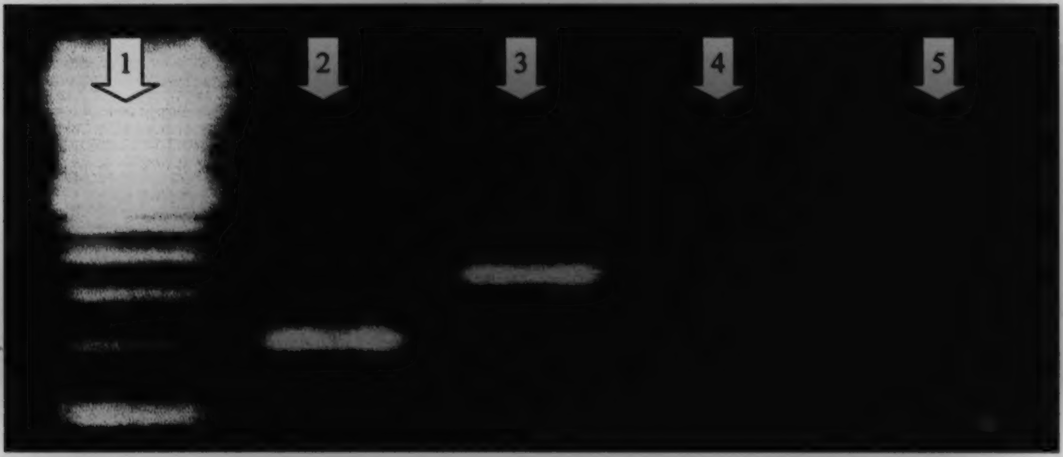

Figure 3.4. PCR amplification products of the piscicolin 126 structural gene in *C. maltaromaticum* UAL26. Primers were chosen at either end of the gene (F2 and R2), or upstream (R1) or downstream (F1), and used in pairs for amplification of genomic DNA. Lane 1: 100 bp DNA ladder, Lane 2: F2 & R2, Lane 3: F1 & R2, Lane 4: F2 & R1, Lane 5: F1 & R1.

3.3.6. Identification of the piscicolin 126 operon and sequencing of the structural gene, promoter region, and induction gene in *C. maltaromaticum* UAL26

The primer pairs listed in Table 3.4 were designed based upon conserved residues or motifs in homologous genes to those found in the piscicolin 126 operon (Figure 3.5). The fragments listed represent amplification between these conserved regions in *C. maltaromaticum* UAL26. The sequences of the structural gene, promoter region, and induction gene showed 100% identity with the sequence of *C. maltaromaticum* JG126 (Gibbs *et al.*, 2000) when compared using the BLAST search tool (NCBI-BLAST, 2004).

Table 3.4. Expected and observed sizes of PCR products using the listed primer pairs to amplify regions of the piscicolin 126 operon in *C. maltaromaticum* UAL26.

Primer pair	Expected size (bp)	Observed size (bp)
U2 & RI	527	~550
FI & RI	296	~300
D1 & D2A	1200	~1150
D1 & D2B	310	~350
FP & RN	393	~400
FN & RN	143	~150
FK2 & RK2	221	~200
FR & RR	403	~425
FT1 & RT1	365	~375
FT1 & RT2	410	~425
FT1 & RE	822	~850
FT2 & RE	429	~425

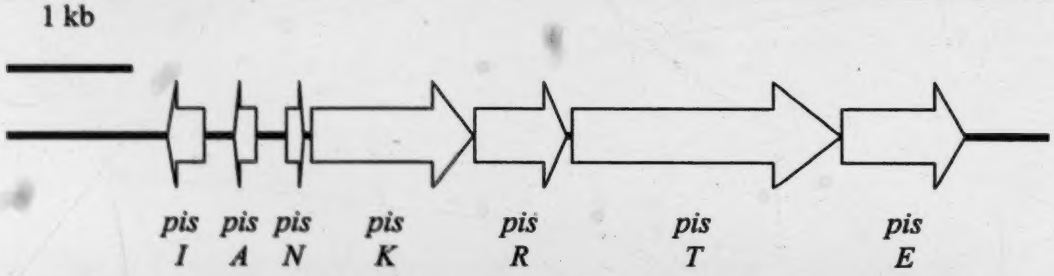

Figure 3.5. Genetic organization of the piscicolin 126 operon, encoding the immunity gene (*pisI*), the structural bacteriocin gene (*pisA*), the induction gene (*pisN*), the histidine kinase gene (*pisK*), the response regulator gene (*pisR*), the dedicated ABC-transporter gene (*pisT*), and the transport accessory gene (*pisE*). Adapted from Gibbs *et al.*, 2000.

3.3.7. Identification of the carnobacteriocin BM1 structural gene in *C. maltaromaticum* UAL26 and JG126

The PCR amplified DNA fragments shown in Figure 3.6 identify the presence of both the piscicolin 126 and carnobacteriocin BM1 structural genes in *C. maltaromaticum* UAL26 and JG126. Amplified fragments are of the expected size for the primers used in the PCR reaction. The carnobacteriocin BM1 operon is shown in Figure 3.7.

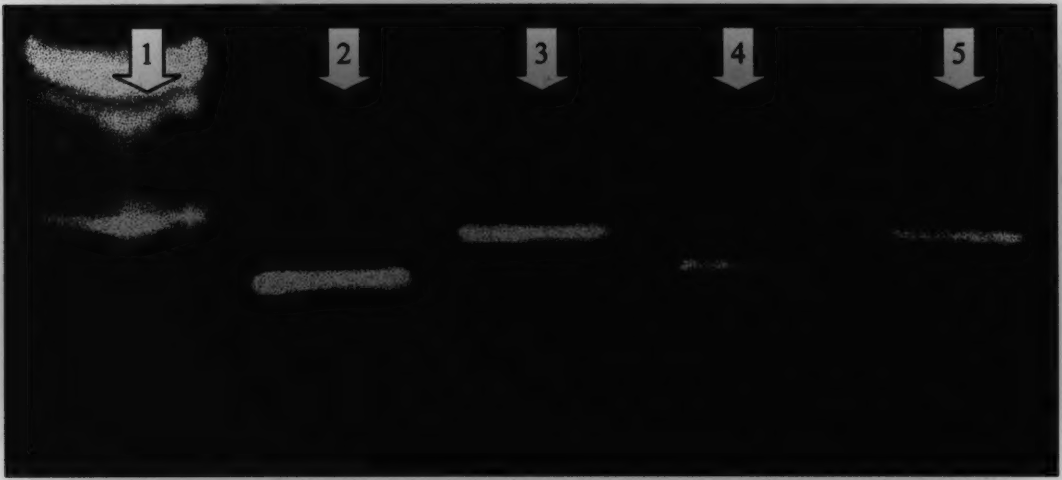

Figure 3.6. PCR amplified bands of the piscicolin 126 and carnobacteriocin BM1 genes in *C. maltaromaticum* UAL26 and JG126. Lane 1: 100 bp marker; lane 2: piscicolin 126 amplified gene product in *C. maltaromaticum* UAL26; lane 3: carnobacteriocin BM1 amplified gene product in *C. maltaromaticum* UAL26; lane 4: piscicolin 126 amplified gene product in *C. maltaromaticum* JG126; lane 5: carnobacteriocin BM1 amplified gene product in *C. maltaromaticum* JG126.

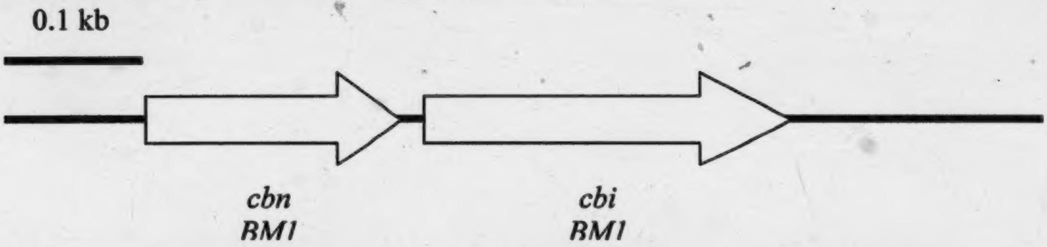

Figure 3.7. Genetic organization of the carnobacteriocin BM1 operon, encoding the bacteriocin structural gene (*cbnBM1*), and the immunity gene (*cbiBM1*). Adapted from Quadri *et al.*, 1994.

3.4. Discussion

The antibacterial activity from C. *maltaromaticum* UAL26 has the characteristics of a typical class IIa bacteriocin. It is proteinaceous, heat-stable, and it is acid tolerant, but it is inactivated at alkaline extremes of pH. These characteristics are common to several other members of the class IIa bacteriocin group.

The XAD-16 resin used during pre-HPLC purification is a non-ionic, aromatic hydrophobic resin. It effectively binds hydrophobic compounds but does not retain highly charged ones. As a general rule, the more hydrophobic the compound, the higher percentage of hydrophobic solvent needed for elution (Mant and Hodges, 1991; Nollet, 2000). Elution of bacteriocin activity required the use of 70% propan-2-ol at pH 2 (which will impart a net positive charge to proteinaceous compounds), which suggested that the bacteriocin from C. *maltaromaticum* UAL26 contains both hydrophobic regions and amino acid residues that are capable of being positively charged. This characteristic made the bacteriocin a prime candidate for further purification by cation exchange. IR120 PLUS resin is a strongly acidic cationic exchange resin. To achieve elution from this resin, a strongly electrolytic hydrophobic solvent (0.1 M NaCl, 50% propan-2-ol) was necessary to interfere with the binding of the bacteriocin to the resin. The C_{18} Mega-Bond pre-HPLC cartridge separates compounds on hydrophobicity, but uses a long linear aliphatic chain to bind the bacteriocin instead of an aromatic matrix as found in the XAD-16 resin. This is a useful pre-HPLC purification due to its ability to remove coloured impurities and various other compounds from the bacteriocin.

RP-HPLC is typically used in a semi-preparative application to purify proteins or peptides for further analytical work, such as mass spectrometry, nuclear magnetic resonance, or amino acid sequencing. While the semi-purified sample of bacteriocin had a single major peak by MALDI-TOF MS analysis, the same sample gave ambiguous results when amino acid sequencing was done. It contained a major sequence and an underlying sequence when PTH-a.a. peak changes were analysed. The major sequence bore no similarity to any known protein in the database; however, the underlying sequence contained similarities to the class IIa bacteriocin protein sequence motif YGNGVXC. As the experimental molecular mass from the MALDI-TOF MS analysis was identical to the reported mass of piscicolin 126 (Jack *et al.*, 1996), DNA primers were designed based upon the published sequence of the piscicolin 126 structural gene. PCR amplification was successful using three pairs of these primers, and the amplified DNA sequence showed that the gene in *C. maltaromaticum* UAL26 was identical to that in *C. maltaromaticum* JG126. The fourth primer pair did not amplify the target DNA sequence, possibly because of problems with primer design and composition.

Isolation of bacteriocins that have already been characterized is not an occurrence new to this study. There are several examples in the literature of isolation of supposedly 'novel' bacteriocins, which, upon further characterization, were found to be homologous to previously reported bacteriocins. In particular, piscicocin V1a (BhugalooVial *et al.*, 1996) is identical in molecular mass to piscicolin 126; piscicolin 61 (Holck *et al.*, 1994) has the same molecular mass as carnobacteriocin A; piscicocin V1b (BhugalooVial *et al.*, 1996) and carnocin CP51 (Herbin *et al.*, 1997) are identical to carnobacteriocin BM1; and carnocin CP52 (Herbin *et al.*, 1997) and the bacteriocin produced by *C. maltaromaticum* A9b (Nilsson *et al.*, 2002) are homologous to carnobacteriocin B2. This occurrence is also not unique to bacteriocins from carnobacteria. Examples have been shown in other

LAB: nisin has been isolated from a variety of *Lactococcus lactis* ssp. *lactis* strains (Hurst, 1981; Harris *et al.*, 1992; Rodriguez *et al.*, 1995), and plantaracin S has been found to be widely distributed in *Lactobacillus plantarum* strains (Maldonado *et al.*, 2002).

Further genetic analysis, using primers based on the sequence of the piscicolin 126 operon in *C. maltaromaticum* JG126, showed that all other genes in the piscicolin 126 operon were present in the genome of *C. maltaromaticum* UAL26. The sequencing of both the bacteriocin structural gene, the promoter region, and the induction gene revealed that there were no genetic differences within these regions.

Lactic acid bacteria commonly carry the genetic elements for production of multiple bacteriocins. *C. maltaromaticum* LV17 (Quadri *et al.*, 1994; Worobo *et al.*, 1994), *Leuconostoc mesenteroides* TA33a (Papathanasopoulos *et al.*, 1997), and *Lc. lactis* subsp. *lactis* biovar *diacetylactis* DPC938 (Morgan *et al.*, 1995) carry the genes for at least three bacteriocins. *Lb. gasseri* LF221 (Majhenic *et al.*, 2004), *Leu. mesenteroides* Y105 (Morisset and Frere, 2002), and *Enterococcus faecium* E13 (Marekova *et al.*, 2003) produce at least two bacteriocins, while several two-component bacteriocins have also been identified (McCormick *et al.*, 1998; Cuozzo *et al.*, 2000; Garneau *et al.*, 2002; Martin *et al.*, 2004). Often, when more than one bacteriocin is produced, they act synergistically, exhibiting a stronger inhibitory effect against sensitive strains when found together (Garneau *et al.*, 2002; Limonet *et al.*, 2004; Martin *et al.*, 2004), although they may also act antagonistically (Mulet-Powell *et al.*, 1998). The identification of the carnobacteriocin BM1 gene in *C. maltaromaticum* UAL26 (Stiles, unpublished data) and in *C. maltaromaticum* JG126 in addition to the piscicolin 126 operon showed that these strains carry the genetic elements for the production of at least two bacteriocins.

The carnobacteriocin BM1 operon in *C. maltaromaticum* LV17B does not contain closely-linked genetic elements for its own export (Quadri *et al.*, 1994; Quadri *et al.*, 1997). It consists of the bacteriocin structural gene and the immunity peptide, and it does not make use of the general secretory pathway. The prebacteriocin gene encodes a leader peptide for dedicated specific export. Because the genetic elements of the plasmid-encoded carnobacteriocin B2 operon are essential for the expression of carnobacteriocin BM1, carnobacteriocin BM1 may make use of the transport machinery encoded by the carnobacteriocin B2 operon in *C. maltaromaticum* LV17B (Quadri *et al.*, 1994; Quadri *et al.*, 1997). As the genetic elements for carnobacteriocin B2 are not present in either *C. maltaromaticum* UAL26 or *C. maltaromaticum* JG126 (results not shown), one possible explanation is that export of carnobacteriocin BM1 in *C. maltaromaticum* UAL26 requires the presence of the piscicolin 126 operon.

In the search for novel bacteriocins to be used for food preservation, lactic acid bacteria are often screened in large numbers for inhibitory activity against food spoilage organisms and foodborne pathogens. The most common source for isolating these bacteria is directly from food. *C. maltaromaticum* UAL26 was isolated from vacuum packaged ground beef in Edmonton, Alberta, Canada (Burns, 1987), while *C. maltaromaticum* JG126 was isolated from spoiled ham in Victoria, Australia (Jack *et al.*, 1996). These two isolates are sufficiently closely related to be classified as the same species, and are similar enough that both carry the genetic elements for production of the same two bacteriocins on their chromosomal DNA. However, having been isolated from two different types of food, and from two geographically distant locations, a certain degree of genetic variation could be expected. The phenomenon of temperature control of bacteriocin production observed in *C. maltaromaticum* UAL26 (Chapter 2, this thesis) is not observed in *C. maltaromaticum* JG126, although the genes of the piscicolin 126 operons are similar in size. *C. maltaromaticum* JG126 produces a detectable amount of bacteriocin in the supernatant at its optimal growth temperature of 25°C when assayed by spot-on-lawn (Jack *et al.*, 1996); however, *C. maltaromaticum* UAL26 only produces enough bacteriocin to be detected in the supernatant at sub-optimal growth temperatures (<19°C; Chapter 2, this thesis). Furthermore, the production of increased titre of bacteriocin in *C. maltaromaticum* UAL26 has a temperature cutoff point which varies depending on the composition of the medium. This indicates that perhaps some difference of control of bacteriocin production exists at either the genetic level, or control of bacteriocin production is related to the expression (mRNA) of the genetic elements of the piscicolin 126 operon.

3.5. References

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4. GENETIC MANIPULATION OF *Carnobacterium maltaromaticum* UAL26

4.1. Introduction

Engineering genetic mutations into a bacteriocin operon can be achieved using several methods. Desired mutations can be made using random mutagenesis (Van der Wal *et al.*, 1998), site-directed mutagenesis (Kazazic *et al.*, 2002), transposon mutagenesis (De Oliveira *et al.*, 1998), or engineering of specific gene fusions (McCormick *et al.*, 1998). Unfortunately, these methods require that the operon be cloned from the strain of origin. In cases where the operon is chromosomally encoded, the use of *in vitro* mutagenesis precludes study of the effects of the mutations in the strain of origin. Instead, *in vivo* mutation techniques were necessary to study the effects of specific gene knockout in *C. maltaromaticum* UAL26.

After the characterization of the piscicolin 126 operon in *C. maltaromaticum* UAL26, it was necessary to determine if other bacteriocins with antilisterial activity were present in the genome. One method of assessing this would be to inactivate the piscicolin 126 structural gene using a double-crossover replacement of the gene with an inactive copy. This procedure has been successfully used previously in lactic acid bacteria (Leenhouts, 1990). A piscicolin 126-negative clone of *C. maltaromaticum* UAL26 would reveal the presence of other bacteriocins produced by the strain, possibly including novel, uncharacterized antibacterial peptides. If other bacteriocin activity was not found, a bacteriocin-negative clone would be useful as a negative control in experiments measuring the antilisterial activity of *C. maltaromaticum* UAL26, including its use in a food system.

Bacteria use specific transport machinery to export proteins into the extracellular medium. Bacterial exporters are proteins with a highly conserved ATP-binding cassette (ABC) region that function to translocate a protein or polypeptide through the cell membrane (Fath and Kolter, 1993). This export function is powered by the hydrolysis of ATP (Fath and Kolter, 1993). Some bacteriocin operons contain a gene which encodes an ABC-transport protein which is specifically dedicated to the task of processing the prebacteriocin into its active form and exporting it from the cell.

The discovery of the presence of the carnobacteriocin BM1 gene in the genome of *C. maltaromaticum* UAL26 (Stiles, unpublished data) precluded the possibility of making a bacteriocin-negative clone without two successive structural gene replacements. It has been suggested that carnobacteriocin BM1 makes use of the transport machinery of carnobacteriocin B2 in *C. maltaromaticum* LV17 for export from the cell (Quadri *et al.*, 1994; Quadri *et al.*, 1997). Because carnobacteriocin B2 is not present in *C. maltaromaticum* UAL26 (unpublished data), it is possible that the machinery of piscicolin 126 performs the same surrogate task. A comparison of the DNA and protein sequences of piscicolin 126, carnobacteriocin BM1, and carnobacteriocin B2 would show the sequence similarity of these proteins. The hypothesis of sharing transport machinery provided the justification for attempts to create a genetic knockout of the piscicolin 126 transporter gene in *C. maltaromaticum* UAL26. A genetic knockout of the transport machinery in *C. maltaromaticum* UAL26 should prevent the prebacteriocins from being exported from the cell, leaving translated prebacteriocin in its unprocessed, inactive form. This should reduce or eliminate the production of bacteriocin by *C. maltaromaticum* UAL26 .

In vivo mutagenesis is a relatively new technique primarily used in eukaryotic genetic manipulation (Storici *et al.*, 2001). The protocol for *in vivo* mutagenesis as an alternative to *in vitro* mutagenesis, could be a quick and simple method to produce a clone of *C. maltaromaticum* UAL26 that is unable to export either piscicolin 126 or carnobacteriocin BM1. However, the limitations of the experimental design precluded using selection by an antibiotic marker gene, and dictated modification and adaptation of the protocol used in eukaryotic organisms. In the event that *in vivo* mutagenesis was unsuccessful because of lack of selection, knocking out the piscicolin 126 transporter or transport accessory gene by Campbell-like integration (Leenhouts, 1990) could also be attempted. The objectives of this research were to alter the piscicolin 126 operon in *C. maltaromaticum* UAL26 with a knockout of the piscicolin 126 structural or transporter gene to make a bacteriocin-negative clone, and to determine if carnobacteriocin BM1 utilizes the transport protein in the piscicolin 126 operon.

4.2. Materials and methods

4.2.1. Bacterial strains and growth media

C. maltaromaticum UAL26 was acquired from the University of Alberta Lactic Acid Bacteria collection (University of Alberta, Edmonton, AB). Stock cultures of *C. maltaromaticum* UAL26 were maintained at -70°C in All Purpose Tween broth (APT; Difco, BD Diagnostics, Sparks, MD) with glycerol (20% vol./vol.; Fisher Scientific, Edmonton, AB). Cultures of *C. maltaromaticum* UAL26 used in this study were prepared by inoculation of cells from frozen stock culture into tempered (by holding the media at 25°C for at least 1 h before inoculation) liquid broth. Cultures were subcultured at least once more at an inoculation volume of 1% vol./vol. before use in experiments.

Escherichia coli MH1 (Casadaban and Cohen, 1980) was acquired from the University of Alberta Food Microbiology culture collection (University of Alberta, Edmonton, AB). Stock cultures of *E. coli* MH1 were maintained at -70°C in Luria Bertani (LB) broth (BD Diagnostics) with glycerol (20% vol./vol.). *E. coli* MH1 carrying the plasmid pGS30 (Vieira and Messing, 1987) was also acquired from the University of Alberta Food Microbiology culture collection. Stock cultures of *E. coli* MH1 pGS30 were maintained at -70°C in LB containing chloramphenicol (25 µg/ml; Fisher Scientific) and glycerol (20% vol./vol.).

4.2.2. Antibacterial activity of *C. maltaromaticum* UAL26

Listeria monocytogenes ATCC 43256 was grown from frozen stock culture in APT broth at 37°C for 24 h, and subcultured at least once before use in experiments as the indicator organism to determine arbitrary activity units (A.U.) in spot-on-lawn assays. Arbitrary activity units were calculated as the reciprocal of the highest dilution in a doubling dilution series that showed a clear zone of inhibition, and are expressed in A.U./ml (Ahn and Stiles, 1990). For the spot-on-lawn assay, 10 µl of each dilution of cell-free (centrifuged at 10,000 x *g* for 5 min), heated (65°C for 30 min) supernatant was spotted onto APT agar plates, and overlayered with 7 ml of soft APT agar (0.75% agar) seeded with a 1% inoculum of *L. monocytogenes* ATCC 43256. Plates were incubated at 37°C for 24 h, and zones of inhibition were measured and A.U./ml calculated.

For the deferred inhibition assay, a 1 µl spot of the *C. maltaromaticum* culture to be assayed was inoculated onto APT agar plates (1.5% agar), allowed to dry, and incubated at 25°C for 1 d. The plates were overlayered with 7 ml of soft APT agar (0.75% agar) seeded with a 1% inoculum of *L. monocytogenes* ATCC 43256. The plates were incubated at 37°C for 24 h and the zones of inhibition were measured. The zone of inhibition was measured as the diameter (mm) across a zone of inhibition of growth of the indicator organism (Tagg *et al.*, 1976)

4.2.3. Construction of the plasmid pLG26X

C. maltaromaticum UAL26 was grown in APT from frozen stock at 25°C for 20 h. The culture (1 ml) was centrifuged (model 5417C; Eppendorf, Hamburg, Germany) at 15,000 x *g* for 3 min, the supernatant discarded, and the cells were resuspended in 1 ml TN150 buffer [10 mM TrisCl (Fisher Scientific), 150 mM NaCl (Fisher Scientific), pH 8]. The cell suspension (1 ml) was transferred into a sterile 2 ml bead-beating tube containing 0.3 g of 0.1 mm silica/zirconium beads (Biospec Products, Bartlesville, OK). The samples were subjected to rotary shaking (Mini Beadbeater; Biospec Products) for 3 min at maximum speed. The tube was cooled on ice for 5 min to allow the beads to settle. The homogenate (500 µl) was transferred into a sterile microcentrifuge tube and extracted by vortexing for 10 s with an equal volume of buffer-saturated phenol. This mixture was separated by centrifuging at 10,000 x *g* for 3 min, and the aqueous layer was transferred to a new microcentrifuge tube. An equal volume of chloroform:isoamyl alcohol (24:1) was added to the tube and separated by centrifuging at 10,000 x *g* for 3 min. The aqueous layer was transferred to a new tube, to which 50 µl of 3 M sodium acetate (Fisher Scientific) pH 5.3 and 1 ml cold (-20°C) 70% ethanol (Fisher Scientific) were added, and the contents of the tube were mixed by vortexing. The mixture was stored for 1 h at -80°C to allow the nucleic acids to precipitate. The tube was centrifuged at 15,000 x *g* for 20 min at 4°C, and the supernatant was removed. The precipitated nucleic acids were redissolved in 500 µl TE buffer [10 mM TrisCl, 1 mM EDTA (Fisher

Scientific), pH 7.5] with 25 µl of RNase A (2 mg/ml; Sigma-Aldrich Canada, Oakville, ON) and the mixture was incubated at 37°C for 1 h. The solution was subjected to a second phenol/chloroform:isoamyl alcohol extraction, precipitation, and centrifugation. The remaining DNA was resuspended in 20 µl TE buffer and stored at -80°C. The purified DNA was diluted 1 in 20 with sterile Milli-Q water before use as template for PCR reactions.

To prepare electrocompetent cells of *C. maltaromaticum* UAL26, a culture was grown from frozen stock in APT broth at 25°C for 20 h. The culture (1 ml) was inoculated into 100 ml of APT broth containing 2% glycine (Fisher Scientific) and incubated at 25°C for 6 h. The culture was centrifuged at 16,000 x *g* at 4°C for 5 min. The supernatant was discarded, and the cells were resuspended in 10 ml of ice-cold sterile Milli-Q water. The centrifugation and resuspension were repeated twice, and the cells were resuspended in 1 ml of electroporation buffer [0.5 M sucrose (Fisher Scientific), 1 mM $MgCl_2$, 5 mM KPO_4 (Fisher Scientific), and 10% glycerol]. These cells were centrifuged at 20,000 x *g* at 4°C for 3 min, the supernatant was discarded, and the pellet was resuspended in 500 µl of electroporation buffer. This volume was split evenly into 5 aliquots and stored at -80°C for further use.

E. coli MH1 pGS30 was subcultured from frozen stock culture in 5 ml of LB broth containing chloramphenicol (25 µg/ml) and grown at 37°C with shaking for 24 h. Template DNA for gene amplification was prepared using the following protocol: cell culture (1 ml) was centrifuged at 20,000 x *g* for 5 min, the supernatant was discarded, and the cell pellet was resuspended in 500 µl sterile Milli-Q water. The cell suspension was centrifuged at 20,000 x *g* for 5 min, resuspended in 100 µl sterile Milli-Q water, and heated at 100°C for 5 min.

E. coli MH1 was made competent for transformation by growing the culture from frozen stock in 5 ml of LB broth at 37°C with shaking for 20 h. The culture (1 ml) was subcultured into 100 ml of fresh LB broth and incubated at 37°C with shaking for 3 h. The culture (10 ml) was aliquoted into 1 ml portions and centrifuged at 20,000 x *g* for 1 min. The supernatant was discarded and 1 ml of sterile, ice-cold 0.1 M $CaCl_2$ (Fisher Scientific) was added. The cells were resuspended using a vortex mixer, and held on ice for 30 min. The tubes were centrifuged at 20,000 x *g* for 1 min. The supernatant was discarded and each pellet was resuspended in 200 µl of 0.1 M $CaCl_2$ with glycerol (20% vol./vol.) and stored at -80°C.

DNA primers, designed based upon the sequence of piscicolin 126 from *C. maltaromaticum* JG126 (Jack *et al.*, 1996), were synthesized on an Applied Biosystems 394 DNA/RNA Synthesizer by the DNA Core Facility (University of Alberta), with a 10 bp sequence at the 5'-end to allow the appropriate restriction endonuclease to cut the amplified DNA fragment. The primers were used in a PCR reaction (Sambrook and Russell, 2001) to amplify the region surrounding the piscicolin 126 structural gene. Two primers were selected at the middle of the coding region for the structural gene (U1 and D1), and the other two primers were selected approximately 1200 bp upstream (D2D) and 850 bp downstream (U2A) of the structural gene. The primer used to amplify the chloramphenicol resistance gene DNA fragment from *E. coli* MH1 pGS30 was designed based upon the sequence of the palindromic multiple cloning site (MCS) containing a *Bam*HI restriction site. The sequence of the primers was as follows:

primer U1 5'-ATATGGATCCGATTGGAGCAAAGCTATTG-3',

primer U2A 5'-AGAGGAGCTCGTTATTAGTGAATTGACAGAA-3',

primer D1 5'-ATATGGATCCTACAGTACAACCATTTTTATTA-3',

primer D2D 5'-AGAGGCATGCATGTTTAAATTTATTTAGCTTTT-3', and

primer CAMR 5'-AAGAATTCCCCGGATCCGT-3'.

For all reactions, DNA was amplified in 100 µl PCR reactions using 0.5 µl of Platinum Taq DNA polymerase High Fidelity (Invitrogen Canada Inc., Burlington, ON), 10 µl of 10 x HIFI Platinum Taq Buffer, 4 µl of 50 mM $MgSO_4$, 1 µl of each primer at 200 nM (pairings of the primers listed above), 2 µl of 10 mM of dNTP solutions (Invitrogen), and 1 µl of purified DNA template isolated from *C. maltaromaticum* UAL26.

The following PCR protocol was used on a GeneAmp PCR system 2400 thermal cycler (version 2.11; Perkin Elmer Corp., Norwalk, CT): 5 min denaturation at 94°C, 40 cycles of: 1 min denaturation at 94°C, 1 min annealing at 50°C, 2 min extension at 68°C, then a final 5 min extension at 72°C, and the samples were cooled and held at 4°C. Each reaction mixture (20 µl) was loaded onto a 1% w./vol. agarose (BD Diagnostics) gel dissolved in 1 x TBE buffer [10.778 g tris(hydroxymethyl)aminomethane (Tris; Fisher Scientific), 0.744 g disodium ethylenediaminetetraacetic acid (EDTA; Fisher Scientific), 5.5 g boric acid (Fisher Scientific)/L] and separated in 1 x TBE buffer by electrophoresis at 110 V for approximately 30 min using a Mini-Sub Cell GT gel box (BioRad Laboratories, Hercules, CA). The gel was visualized with a Gel Doc 1000 UV transilluminator (BioRad) and photographed.

To prepare stock amounts of plasmid pGS30, a maxiprep (Sambrook and Russell, 2001) of *E. coli* MH1 pGS30 was performed and purified by cesium chloride (CsCl) gradient ultracentrifugation. DNA (6 ml) from the maxiprep was used to dissolve 6 g of CsCl in a plastic ultracentrifuge tube. Ethidium bromide (300 µl; BioRad) was added, and centrifuged in an ultracentrifuge (model L8-M; Beckman Coulter, Fullerton, CA) at 250,000 x *g* under vacuum for 20 h. The tube containing the centrifuged DNA was examined for the presence of plasmid DNA under UV light at 260 nm (UV transilluminator; Fotodyne, Hartland, WI). Plasmid DNA was transferred into a plastic tube by glass pipette. The ethidium bromide was extracted from the plasmid DNA sample four times by mixing 4 ml of isoamyl alcohol with the DNA solution, inverting the tube 20 times, and removing the isoamyl alcohol layer. The DNA solution was put into dialysis tubing (Spectra/por 6000 to 8000 Da cutoff; Spectrum Laboratories, Rancho Dominguez, CA), and dialysed in 500 ml of TE buffer for 3 h, changing the buffer at hourly intervals. After dialysis, the DNA solution was transferred into a microcentrifuge tube, and extracted 3 times with phenol/chloroform:isoamyl alcohol as described above. The purified DNA was precipitated by adding 0.1 volume of 3 M sodium acetate (Fisher Scientific) and 2 volumes of 100% ethanol (Fisher Scientific), and the solution was stored at -20°C for 1 h. The precipitated DNA was centrifuged at 20,000 x *g* at 4°C for 10 min, and the supernatant was removed. The DNA pellet was washed with 500 µl of 70% ethanol. After removing the final volume of ethanol, the purified DNA was allowed to dry for 15 min and then redissolved in 500 µl of TE buffer.

DNA fragments from PCR amplification were cleaned using a QiaQuick PCR purification columns (Qiagen Inc., Hilden, Germany), eluted with a final volume of 50 µl

EB buffer (10 mM TrisCl, pH 8.5), and then cut sequentially with the appropriate restriction endonuclease(s). Purified DNA fragments (40 µl) were added to 5 µl of 10 x React 1 restriction enzyme buffer (Invitrogen), and 5 µl of the first restriction enzyme (Invitrogen) was added according to Table 4.1. For the pUC118 plasmid (kindly provided by Dr. Marco van Belkum; Canbiocin, University of Alberta), 20 µl of CsCl gradient purified pUC118 plasmid DNA was added to 2.5 µl of the restriction enzyme buffer (React 1), and then 2.5 µl of each restriction enzyme was added. The mixtures were incubated at 37°C for 3 h, repurified by phenol/chloroform:isoamyl alcohol purification as described above, concentrated using a QiaQuick PCR purification column, and eluted with 40 µl of Milli-Q water. The process was repeated using the second set of restriction enzymes and the DNA was cleaned using a QiaQuick PCR purification column and eluted with 50 µl of Milli-Q water.

Table 4.1. Summary of restriction enzymes used to cut PCR-amplified DNA fragments and pUC118 plasmid DNA.

	Restriction enzymes	
DNA fragment	First enzyme	Second enzyme
U1:U2A	*Sst*I	*Bam*HI
D1:D2D	*Bam*HI	*Sph*I
CAMR	*Bam*HI	N/A
pUC118	*Sst*I	*Sph*I

Ligation was done by adding 6 µl of sterile Milli-Q water and 4 µl of 5 x ligation buffer (Invitrogen) to 2 µl of each piece of cut PCR-amplified DNA. T4 DNA ligase (2 µl; Invitrogen) was added, and the solution mixed using a vortex. This solution was incubated at 25°C for 1 h, and held in a water bath at 4°C for 24 h. The ligation mixture (5 µl) was loaded onto a 1% w./vol. agarose (BD Diagnostics) gel (dissolved in 1 x TBE buffer), separated by electrophoresis, and visualized as described above. A diagrammatic representation of the intended pLG26X plasmid construct is shown in Figure 4.1.

Ligated DNA (15 µl) was added to 200 µl of competent *E. coli* MH1 cells, mixed by vortex, and held on ice for 30 min. The mixture was incubated at 37°C for 5 min in a block heater. LB broth (500 µl) was added and the culture was incubated at 37°C for 1 h. The culture was plated in 100 µl portions onto LB agar plates with chloramphenicol (20 µg/ml) to select for resistant colonies. Resistant colonies that grew within 48 h were picked from the agar plates, and grown in LB broth containing chloramphenicol (20 µg/ml) at 37°C with shaking for 24 h, supplemented with glycerol (20% vol./vol.) and frozen at -80°C as stock cultures.

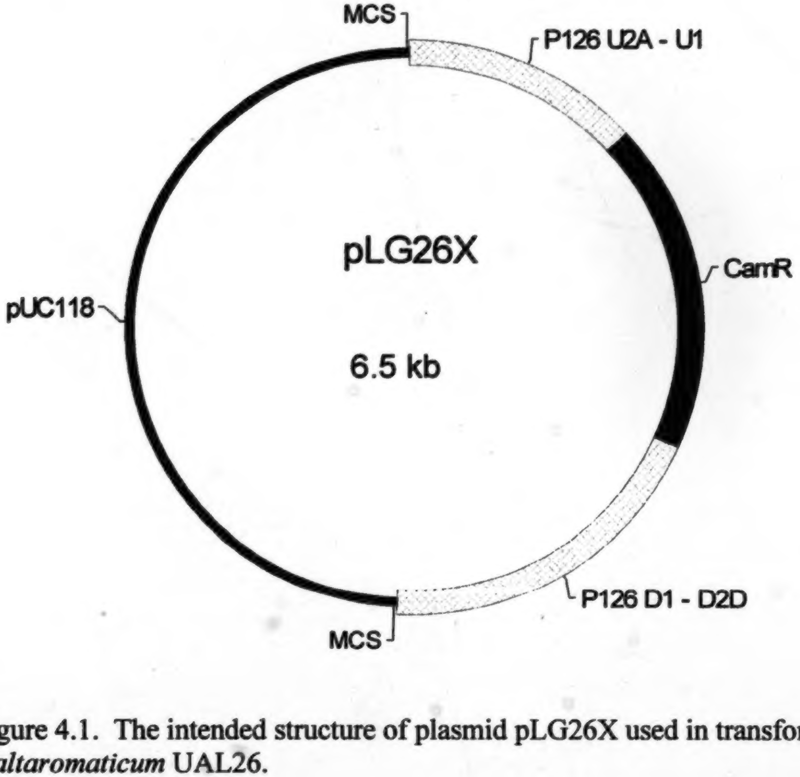

Figure 4.1. The intended structure of plasmid pLG26X used in transformation of *C. maltaromaticum* UAL26.

Minipreps (Sambrook and Russell, 2001) of plasmid DNA from *E. coli* MH1 pLG26X isolates were made from stock cultures grown in LB broth containing 20 µg/ml chloramphenicol at 37°C with shaking for 24 h. Each cell culture (1 ml) was centrifuged at 20,000 x *g* for 5 min, the supernatant was discarded, and the cells were resuspended in 250 µl ice-cold STE (10 mM TrisCl pH8, 0.1 M NaCl, and 1 mM EDTA, pH 8) buffer. The centrifugation was repeated and the cells were lysed by the alkaline lysis method (Sambrook and Russell, 2001) and extracted by phenol/chloroform:isoamyl alcohol extraction as described above. Purified DNA of pLG26X (20 µl) was cut with the appropriate restriction enzymes, incubated for 1 h, loaded onto a 1% w./vol. agarose (Becton Dickinson) gel, separated by electrophoresis, and visualized as described above.

A maxiprep (Sambrook and Russell, 2001) of plasmid DNA from an *E. coli* MH1 pLG26X transformant was done and then purified by CsCl gradient ultracentrifugation as described above.

To transform *C. maltaromaticum* UAL26 with pLG26X, purified pLG26X plasmid DNA, electrocompetent *C. maltaromaticum* UAL26 (100 µl), and an electroporation cuvette (BioRad) were placed on ice. Plasmid DNA (10 µl) was added to the electrocompetent cells and mixed briefly. The mixture was transferred into the electroporation cuvette and held on ice for 2 min, then transformed using a Gene Pulser Electroporator (BioRad) at 1 kV and 800 Ω resistance. APT (1 ml) supplemented with 0.5 M sucrose and 20 mM $MgCl_2$ was added to the cuvette and the mixture was transferred to a microcentrifuge tube. The culture was incubated at 25°C for 4 h, plated in 100 µl volumes onto APT agar plates containing chloramphenicol (20 µg/ml), and incubated at 25°C. Colonies that grew within 6 d were picked from the agar plates, grown in APT broth with chloramphenicol (20 μg/ml) at 25°C for 24 h, supplemented with glycerol (20% vol./vol.), and stored at -80°C as stock cultures.

To determine if Campbell-like integration using pLG26X was successful, the chromosomal DNA from *C. maltaromaticum* UAL26 isolates transformed with pLG26X was evaluated for integration of the plasmid using PCR. For all reactions, DNA was amplified in 100 μl PCR reactions using 0.5 μl of recombinant Taq DNA polymerase (Invitrogen), 10 μl of 10 x Taq Buffer, 4 μl of 50 mM $MgCl_2$, 1 μl of each primer at 200 nM (primers FI and RP), 2 μl of 10 mM of dNTP solutions (Invitrogen), and 1 μl of DNA template isolated from three *C. maltaromaticum* UAL26 pLG26X transformants, or from wildtype *C. maltaromaticum* UAL26, prepared using the following protocol: cell culture (1 ml) was centrifuged at 20,000 x *g* for 5 min, the supernatant was discarded, and the cell pellet was resuspended in 500 μl of sterile Milli-Q water. The cells were centrifuged again at 20,000 x *g* for 5 min, resuspended in 100 μl sterile Milli-Q water, and heated at 100°C for 5 min.

The following PCR protocol was used on a GeneAmp PCR system 2400 thermal cycler: hot start addition of Taq polymerase (D'Aquila *et al.*, 1991; Erlich *et al.*, 1991; Mullis, 1991), 2 min denaturation at 94°C, 40 cycles of: 1 min denaturation at 94°C, 1 min annealing at 50°C, 2 min extension at 68°C, and a final 5 min extension at 72°C. The samples were cooled and held at 4°C. Each reaction mixture (25 μl) was loaded onto a 1% w./vol. agarose gel, separated by electrophoresis, and visualized as described above.

Upon failure to isolate any transformant colonies of *C. maltaromaticum* UAL26 that had integration of the pLG26X plasmid when transformed, pLG26X plasmid template was sent to the Core DNA Facility (University of Alberta) where the DNA was sequenced by automated capillary electrophoresis (CEQ2000XL DNA Analysis System; Beckman Coulter) using universal primers supplied by the sequencing facility. DNA sequencing was done using the Sanger dideoxynucleotide termination sequencing protocol. Sequencing reactions were prepared by thermocycler amplification, separated by electrophoresis, and analysed as described above.

4.2.4. Direct transformation of electrocompetent *C. maltaromaticum* UAL26 with ligated PCR products

Competent *C. maltaromaticum* UAL26 was transformed using a linear construct similar to the insert in pLG26X, with a smaller upstream fragment, and without being inserted into a plasmid vector. The upstream fragment was approximately 600 bp in length, using the primers U1 and U2C, of the following sequence:

primer U2C: 5'-AGAGCCCGGGCTAATATCCATATCTAATATTG-3'.

PCR and agarose gel electrophoresis were done according to the protocol described in section 4.2.3. The DNA products from PCR were cut with *Bam*HI at 37°C for 3 h, cleaned by QiaQuick column, and ligated according to the protocol described in section 4.2.3.

Electrocompetent *C. maltaromaticum* UAL26 cells were transformed with the protocol described in section 4.2.3 at a setting of 2.5 kV and 200 Ω resistance. The culture was incubated at 25°C for 6 h, plated in 100 µl volumes onto APT agar plates containing chloramphenicol (20 µg/ml), and incubated at 25°C. A diagrammatic representation of the intended construct is shown in Figure 4.2.

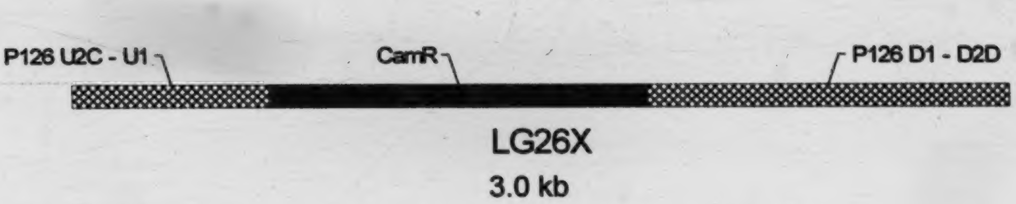

Figure 4.2. The structure of the linear construct LG26X used for direct transformation of *C. maltaromaticum* UAL26.

Colonies of transformed *C. maltaromaticum* UAL26 that grew in 6 d or less on APT agar plates containing chloramphenicol (20 μg/ml) were selected and transferred to APT agar plates to test for activity by deferred inhibition using *L. monocytogenes* ATCC 43256 as the indicator organism. The plates were incubated at 15°C for 48 h for growth of the producer organism and at 37°C for 24 h for growth of the indicator organism.

Ten colonies that had the fastest growth but failed to inhibit *L. monocytogenes* ATCC 43256 were subcultured into APT broth with chloramphenicol (20 μg/ml) and grown at 15°C for 48 h to test for chloramphenicol resistance. These ten cultures were also subcultured into APT broth without chloramphenicol and assayed by the spot-on-lawn assay using *L. monocytogenes* ATCC 43256 as the indicator organism.

The chromosomal DNA from *C. maltaromaticum* UAL26 isolates transformed with LG26X was evaluated for integration of the linear DNA using PCR. PCR was done on the direct transformants using the protocol described in section 4.2.3.

4.2.5. Comparison of the genetic and protein sequences of the structural genes of carnobacteriocin BM1, carnobacteriocin B2, and piscicolin 126, and the piscicolin 126 and carnobacteriocin B2 ABC-transporters

To evaluate DNA and protein sequence similarity between the piscicolin 126, carnobacteriocin BM1, and carnobacteriocin B2 prebacteriocins, mature bacteriocins, and leader sequences, and the piscicolin 126 and carnobacteriocin B2 transporter genes, comparisons of the gene sequences from *C. maltaromaticum* LV17B (Stiles, 1995; Quadri *et al.*, 1997) and *C. maltaromaticum* JG126 (Jack *et al.*, 1996; Wettenhall *et al.*, 2000) were made using the DNAStar program (DNAStar Inc., Madison, WI.) using the ClustalW alignment method (Slow/Accurate, IUB settings for DNA sequences, Slow/Accurate, Gonnet settings for translated protein sequences).

4.2.6. *In vivo* mutagenesis of the piscicolin 126 transport gene in *C. maltaromaticum* UAL26

To produce a genetic knockout of the transporter gene in *C. maltaromaticum* UAL26, a protocol for *in vivo* mutagenesis was designed based on a protocol used in eukaryotic cells (Storici *et al.*, 2001). A 99-bp single stranded DNA oligomer was designed with identical sequence to the piscicolin 126 transporter gene (Gibbs *et al.*, 2000) except for two specific bp insertions at either end of the oligonucleotide. This oligonucleotide sequence, named LGPISTM, containing the insertion mutations is shown in Figure 4.3.

5'-ATGAAACAAT**G**TTACTCATATTCAGCAACAAGATGAAAAAGATTGTGGT
GTAGCATGTATAGCAATGATTTTAAAACATTACAAAACAG**C**AAATTCCCA-3'

Figure 4.3. Base-pair insertions in oligonucleotide LGPISTM. The two insertions are marked in bold, underlined letters. The remaining sequence is derived from the *PisT* gene in *C. maltaromaticum* JG126 (Gibbs *et al.*, 2000).

Two primers of 20 bp length complementary to the 99-bp oligonucleotide were also synthesized:

primer PISTF: 5'-ATGAAACAATGTTACTCATA-3', and

primer PISTR: 5'-TGGGAATTTGCTGTTTTGTA-3'.

These primers were used to amplify the 99-bp oligonucleotide template by PCR according to the protocol described in section 4.2.3.

The purified PCR amplified 99-bp oligomer was purified with a QiaQuick purification column (Qiagen), heated at 100°C for 2 min to denature the double-stranded DNA, and placed on ice to cool for 2 min. A mixture of the PCR-amplified DNA (10 µl), and a tube of electrocompetent *C. maltaromaticum* UAL26 (100 µl) was electroporated as described in section 4.2.4. APT (1 ml) supplemented with 0.5 M sucrose and 20 mM $MgCl_2$ was added to the electroporation cuvette and the mixture transferred to a new microcentrifuge tube. The culture was incubated at 25°C for 24 h and serially diluted 1:100 three times, giving 10^{-2}, 10^{-4}, and 10^{-6} dilutions. Portions of the 10^{-6} dilution (100 µl) were plated onto APT agar plates, incubated at 15°C for 48 h, and stored at 4°C for the remainder of the experiment. A total of 950 colonies were removed from the plates using a sterile wire and inoculated into fresh APT agar plates in duplicate. These plates were incubated at 15°C for 48 h, and one of the two duplicate plates was overlayered with 7 ml of soft APT agar that was seeded with a 1% inoculum of *L. monocytogenes* ATCC 43256. This plate was incubated at 37°C for 24 h. Colonies that had little or no activity against the indicator were isolated from the corresponding duplicate plate, grown on a new APT agar plate and overlayered using the same method. The colonies were also inoculated and grown in 10 ml of APT broth at 15°C for 24 h, and then assayed by a spot-on-lawn assay.

One colony of *C. maltaromaticum* UAL26 was isolated that had potentially incorporated the 99-bp oligomer (referred to as *C. maltaromaticum* 35-19). *C.*

maltaromaticum 35-19 was grown on APT agar at 15°C and 25°C and assayed for antibacterial activity by deferred inhibition.

To determine if the engineered mutations were present in *C. maltaromaticum* 35-19, template for DNA sequencing from isolate *C. maltaromaticum* 35-19 was prepared by the boiling method described in section 4.2.3. The DNA was sent for sequencing with the following primers:

primer LG3519F: 5'-TGAGTCTGACTTAGTAAGGC-3', and

primer LG3519R: 5'-CATCGTTATCAGCTTGGATAG-3'.

The DNA was sequenced by automated capillary electrophoresis (CEQ2000XL DNA Analysis System; Beckman Coulter; DNA Core Facility, University of Alberta) as described in section 4.2.3.

4.2.7. Construction of the plasmid pLGPISTM

DNA primers (Applied Biosystems 394 DNA/RNA Synthesizer, DNA Core Facility, University of Alberta) were designed based upon the sequence of the piscicolin 126 transport gene from *C. maltaromaticum* JG126 (Jack *et al.*, 1996), with a 10 bp sequence at the beginning (5'-end) to allow the appropriate restriction endonucleases to cut the PCR amplified DNA fragment. The primers were used in a PCR reaction (Sambrook and Russell, 2001) to amplify a region within the piscicolin 126 transport gene. The primers used to amplify the fragment were designed to contain the *Bam*HI and *Sph*I restriction sites at either end of the amplified region. The sequence of the primers was as follows:

primer LG26PTF1 5'-AGAGGAGCTCCATATTCAGCAACAAGATGA-3', and

primer LG26PTR2 5'-ACACGGATCCAATGTTGCACCAGCAACC-3'.

PCR and agarose gel electrophoresis were done as described in section 4.2.3, but using an annealing temperature of 60°C.

PCR amplified fragments were cleaned using QiaQuick PCR purification columns, eluted with a final volume of 35 µl, and then digested twice with the appropriate restriction endonucleases (*Bam*HI and *Sph*I in 10 x React 1 buffer, each 10% vol./vol.). Plasmid vector pUC118 was cut using the same protocol, without the QiaQuick purification step. The mixtures were incubated at 37°C for 3 h and then subjected to the phenol/chloroform:isoamyl alcohol purification as described in section 4.2.3, and precipitated using 0.1 vol./vol. of 3 M sodium acetate (pH 5.2) and 2 vol./vol. of 100% ethanol at -20°C for 24 h. The precipitated DNA was centrifuged at 20,000 x *g* at 4°C for 10 min. The supernatant was removed, the precipitate washed briefly in 75% ethanol, and resuspended in a total volume of 35 µl of Milli-Q water for ligation. Ligation was achieved by adding 10 µl of 5 x ligation buffer and 5 µl of T4 DNA ligase enzyme. The solution was mixed by vortexing and incubated at 25°C for 1 h.

Ligated DNA (50 µl) was added to 200 µl of competent *E. coli* MH1 cells, mixed by vortex, and incubated on ice for 30 min. The tube was incubated at 37°C for 5 min in a block heater. LB broth (500 µl) was added and the culture incubated at 37°C for 1 h. The culture was plated in 100 µl portions onto LB agar plates supplemented with erythromycin (200 µg/ml; Fisher Scientific) to select for resistant colonies. Colonies that grew in 72 h or less were picked from the agar plates, grown in LB broth with erythromycin (200 µg/ml) at 37°C with shaking for 24 h. Cell cultures (1 ml) were centrifuged at 20,000 x *g* for 5 min, the supernatant was discarded, and the cells were resuspended in 250 µl of ice-cold STE (10 mM TrisCl, 0.1 M NaCl, and 1 mM EDTA, pH 8) buffer. The centrifugation was repeated and the cells were lysed by the alkaline lysis method (Sambrook and Russell, 2001), and extracted using the phenol/chloroform:isoamyl alcohol extraction method described in section 4.2.3. Purified pLGPISTM DNA was cut with the appropriate restriction enzymes and incubated for 1 h, then loaded onto a 1% w./vol. agarose gel, separated by electrophoresis, and visualized as described in section 4.2.3. Those cultures which showed the correct sized insert were supplemented with glycerol (20% vol./vol.) and frozen at -80°C as stock cultures.

A maxiprep of an *E. coli* MH1 pLGPISTM transformant was performed (Sambrook and Russell, 2001) and the DNA was purified by CsCl gradient ultracentrifugation as described in section 4.2.3.

The pLGPISTM plasmid DNA was used to transform electrocompetent *C. maltaromaticum* UAL26 according to the protocol described in section 4.2.4. The culture was incubated at 25°C for 4 h and then plated in 100 µl volumes onto APT agar plates supplemented with erythromycin (5 µg/ml) and incubated at 25°C. Resistant colonies that grew within 10 d were picked from the agar plates, and assayed by deferred inhibition.

4.2.8. Construction of the plasmids pLGPISKM, pLGPISRM, and pLGPISEM

Three plasmids, pLGPISKM, pLGPISRM, and pLGPISEM were prepared following the above PCR protocol using the following primer pairs:

primer PISKMF1 5'-AGAGGAGCTCAAGCGTACTTACTAAGAATG-3',

primer PISKMR2 5'-ACACGGATCCTTACCAAATAAGGATAATGC-3',

primer PISRMF1 5'-AGAGGAGCTCTGCGAAGATCAAATCATTCA-3', primer PISRMR2 5'-ACACGGATCCCTTTAATTTGCTTTGCCTTAC-3',

primer PISEMF1 5'-AGAGGAGCTCATGCATAATAATAACTGGCG-3', and

primer PISEMR2 5'-ACACGGATCCTTATTCTTGATTTAGCAACAC-3'.

The plasmids were cut, ligated, transformed into competent *E. coli* MH1, characterized, and purified by large scale maxiprep following the protocol used for pLGPISTM.

C. maltaromaticum UAL26 was transformed with pLGPISEM plasmid DNA following the protocol described in section 4.2.4.

4.3. Results

4.3.1. Evaluation of *C. maltaromaticum* UAL26 transformants for double crossover gene replacement of the piscicolin 126 structural gene

Isolated DNA of pLG26X from an *E. coli* MH1 transformant was used to transform electrocompetent *C. maltaromaticum* UAL26, and the resultant colonies were analysed by PCR to determine the presence of the piscicolin 126 structural gene. All transformants analysed by PCR showed that amplification of the piscicolin 126 structural gene was of identical size to the untransformed *C. maltaromaticum* UAL26 (approximately 950 bp band using primers FI and RP; data not shown). No transformants containing the correct insert were isolated. To determine why no transformants containing the correct insert were found, the purified plasmid DNA from one of the three *E. coli* MH1 pLG26X transformants was sent for DNA sequencing and a deletion in the D1-D2D region was revealed (Figure 4.4).

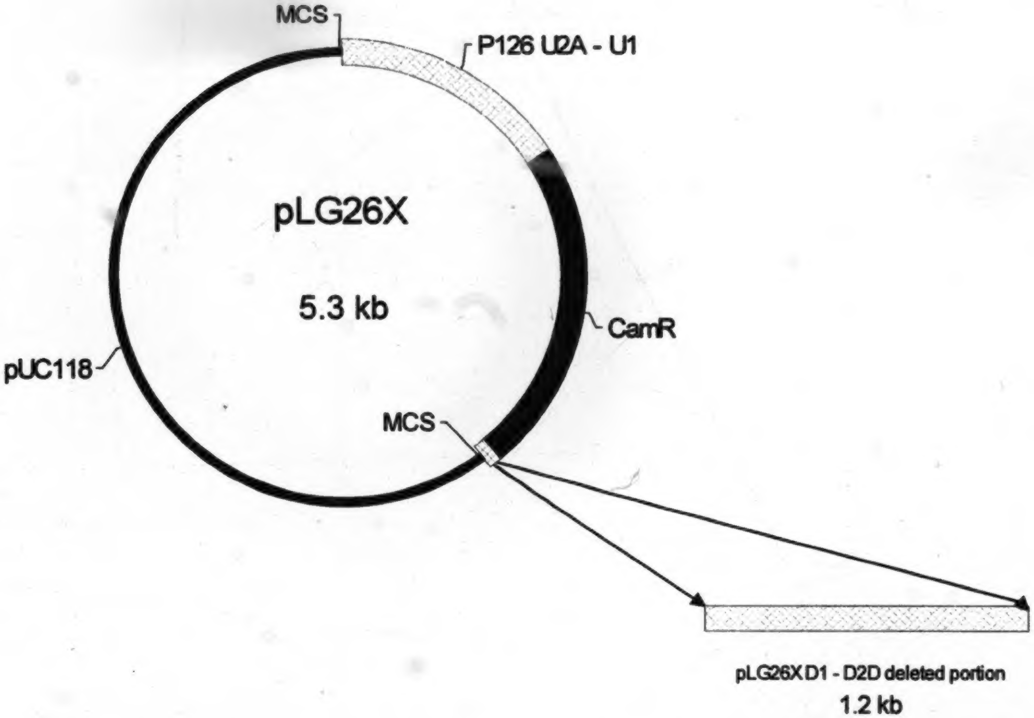

Figure 4.4. The resultant structure of pLG26X from the transformed *E. coli* MH1 host.

4.3.2. Evaluation *C. maltaromaticum* UAL26 direct transformants for double crossover gene replacement of the piscicolin 126 structural gene

The ligated PCR fragments (without pUC118) were used to directly transform *C. maltaromaticum* UAL26. Ten of the resultant colonies were analysed by PCR amplification of the piscicolin 126 structural gene. All transformants analysed by PCR showed that amplification of the piscicolin 126 structural gene was of identical size to untransformed *C. maltaromaticum* UAL26. All isolates grew in the presence of chloramphenicol, and showed inhibition of *L. monocytogenes* ATCC 43256 in deferred inhibition tests. Six of ten isolates produced detectable activity by the spot-on-lawn assay.

4.3.3. Comparison of the genetic and protein sequences of the structural genes of carnobacteriocin BM1, carnobacteriocin B2, and piscicolin 126, and the piscicolin 126 and carnobacteriocin B2 ABC-transporters

A comparison of the DNA and protein sequences of the three bacteriocins and two transport proteins is shown in Figure 4.5. There is a high percentage of identity between the DNA sequences of piscicolin 126, carnobacteriocin BM1, and carnobacteriocin B2, particularly in the leader portion (~70%), but less so in the remainder (mature portion) of the genes. The translated protein sequence shows similar results, except that the entire protein sequence is slightly higher in overall identity between the bacteriocins (~40%) than in the mature portion of the peptide (~30 to 35%); however, the leader portions share strong identity in protein sequence (~60 to 70%). The DNA and protein sequences of the piscicolin 126 and carnobacteriocin B2 transporters are over 70% identical, indicating that these proteins are highly homologous.

4.3.4. *In vivo* mutagenesis of the piscicolin 126 transport gene in *C. maltaromaticum* UAL26

Of the 950 colonies screened for loss of antilisterial activity, only one had reduced activity in a deferred inhibition assay using *L. monocytogenes* ATCC 43256 as the indicator organism. This mutant, *C. maltaromaticum* 35-19, did not have the specific mutations engineered into the 99-bp oligonucleotide fragment used in the transformation, as verified by DNA sequencing of the 99-bp region in the chromosomal DNA of *C. maltaromaticum* 35-19.

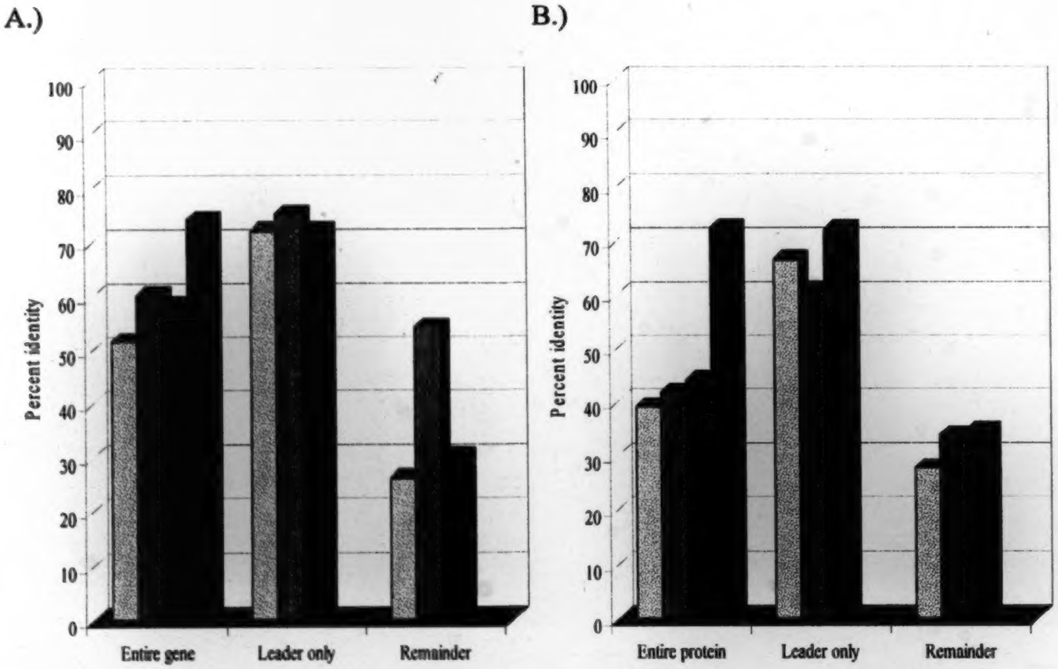

Figure 4.5.A.) Comparison of the DNA sequence of the piscicolin 126 (P126), carnobacteriocin BM1 (BM1), and carnobacteriocin B2 (B2) structural genes, and the P126 and B2 transporter genes, and B.) Comparison of the protein sequence of the P126, BM1, and B2 structural proteins, and the P126 and B2 transporter proteins. P126 to BM1 (▨); P126 to B2 (■); BM1 to B2 (■); and P126 transport to B2 transport (■).

4.3.5. Transformation of electrocompetent *C. maltaromaticum* UAL26 with the plasmids pLGPISTM and pLGPISEM

The transformation of *C. maltaromaticum* UAL26 with pLGPISTM plasmid DNA resulted in the growth of 18 colonies. None of the colonies had reduced antibacterial activity when assayed by deferred inhibition using *L. monocytogenes* ATCC 43256 as the indicator organism. The transformation of *C. maltaromaticum* UAL26 with pLGPISEM plasmid DNA resulted in the growth of 24 colonies. One colony from transformed *C. maltaromaticum* UAL26 had reduced antibacterial activity when assayed by deferred inhibition using *L. monocytogenes* ATCC 43256 as the indicator organism.

4.4. Discussion

Efforts to transform *C. maltaromaticum* UAL26 with a modified version of the piscicolin 126 structural gene were unsuccessful, both when indirectly amplified in an *E. coli* host, and by direct transformation. After the discovery of the carnobacteriocin BM1 gene in the *C. maltaromaticum* UAL26 genome, it became clear that a successful knockout of the piscicolin 126 structural gene would not be sufficient to create a bacteriocin negative strain, and another successive knockout of the carnobacteriocin BM1 structural gene would also be necessary. Instead of attempting this, the focus was shifted to knocking out the piscicolin 126 transporter gene, which should also create a bacteriocin-negative strain.

With the hypothesis that carnobacteriocin BM1 may make use of the transport machinery of carnobacteriocin B2 for export from the cell (Quadri *et al.*, 1994), comparisons of the DNA and protein sequences of different activity domains of the three bacteriocins (leader peptide, active bacteriocin), and comparisons of the carnobacteriocin B2 and piscicolin 126 transporter genes were done. The high degree of identity found between the bacteriocin leader sequences suggests that carnobacteriocin BM1 may use the piscicolin 126 transport machinery as a surrogate export pathway, in a manner homologous to that hypothesized in *C. maltaromaticum* LV17 (Quadri *et al.*, 1994). Proof of this hypothesis required the knockout of the piscicolin 126 transporter gene. Achieving a successful knockout was attempted *in vivo* with the *C. maltaromaticum* UAL26 strain, because it contains both bacteriocin operons on the genome. Unfortunately, of the methods attempted, none resulted in indisputable proof of the desired knockout. Two isolates showed reduced activity, but failed to entirely eliminate activity against the indicator strain. Explanation for the reduced activity remains undetermined. The isolate from the *in vivo* mutagenesis showed only a small diameter zone of inhibition (~4 mm), much smaller than the wildtype culture grown under the same conditions (results not shown). The isolate from the transformation with pLGPISEM showed a similar reduction in activity, however, it was later determined that the transport accessory gene fragment inserted into plasmid pLGPISEM should contain a functional copy of the *pisE* gene, therefore Campbell-like integration should not result in altered gene function.

A strain of *Propionibacterium jensenii*, that produces the bacteriocin propionicin T1, was evaluated for the presence of a gene in the bacteriocin operon with high homology to known ABC-transporters (Faye *et al.*, 2004). The strains that carried the gene showed normal bacteriocin expression; however, three strains in which the transporter gene was absent had reduced or completely eliminated bacteriocin activity. The reduction in antimicrobial activity by these strains suggests that the transporter gene is potentially necessary for proper propionicin T1 activity (Faye *et al.*, 2004). The results of the *P. jensenii* study may be related to the reduced antibacterial effect observed in *C. maltaromaticum* UAL26 isolates created by the *in vivo* mutagenesis and Campbell-like integration experiments in this study. Further study of the reason for reduced antimicrobial activity against the *L. monocytogenes* indicator strain is necessary in both cases.

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5. EFFECT OF TEMPERATURE AND INDUCTION ON BACTERIOCIN EXPRESSION IN *Carnobacterium maltaromaticum* UAL26

5.1. Introduction

Production of bacteriocins is often regulated by specific requirements and expression is not always constitutive. Instead, a particular growth condition, a component of the medium, or a growth factor may be required to induce bacteriocin production. For example, lactacin 481 production by *Lactococcus lactis* is dependent on an acidic pH and involves transcription of the required genes for bacteriocin production, regulation, and export under the control of two promoter regions. These promoter regions are activated by production of lactic acid by the producer strain, or by artificial acidification of the growth medium (Hindre *et al.*, 2004). In the case of the lantibiotic nukacin ISK-1, produced by *Staphylococcus warneri* ISK-1, bacteriocin production can be increased by increasing the osmolarity of the growth medium by addition of sodium chloride. This increase in bacteriocin production by higher salt concentrations is the result of increased transcription of the bacteriocin structural gene (Sashihara *et al.*, 2001). A complex mechanism of induction, involving proteolytic cleavage of an inducer peptide in the extracellular medium, is required for transcriptional activation of the bacteriocin operons for production of ruminococcin A by *Ruminococcus gnavus* E1 (Gomez *et al.*, 2002).

With the knowledge that bacteriocin production in *C. maltaromaticum* UAL26 is temperature dependent (Chapter 2, this thesis), the next logical step was to elucidate the nature of regulation of bacteriocin production. It is possible that control of bacteriocin production in *C. maltaromaticum* UAL26 is either a result of genetic influences within the piscicolin 126 operon, controlled during expression, during translation, by posttranslational modification, by enzymatic action, or possibly by some other cellular process.

It is possible that production of piscicolin 126 and carnobacteriocin BM1 by *C. maltaromaticum* UAL26 (Chapter 3, this thesis) is regulated by the same cellular process. The most likely of the above possibilities is at the genetic level of the piscicolin 126 operon or during expression (mRNA). Any difference in control at the genetic level would most likely be evident in the promoter region. This was evaluated by comparison of the promoter region sequence to that found in *C. maltaromaticum* JG126 (Chapter 3, this thesis). Comparison showed no difference in these DNA sequences. It was discovered that *C. maltaromaticum* JG126 also carries the genes for carnobacteriocin BM1 in addition to those for production of piscicolin 126, but was not subject to the same temperature control of bacteriocin production observed in *C. maltaromaticum* UAL26, therefore *C. maltaromaticum* JG126 can be used as a reference strain in experiments. Regulation of gene expression was the next most likely means of explaining the effect of temperature on bacteriocin production in *C. maltaromaticum* UAL26.

Because mRNA species may be very short lived (from less than 1 min up to 20 min) during production of certain proteins (Grunberg-Manago, 1999; Rauhut and Klug, 1999; Regnier and Arraiano, 2000), comparing the amount of mRNA transcribed from the bacteriocin structural genes requires extraction of RNA samples when mRNA levels for these genes would be fully induced. Isolation of sufficient quantities of mRNA requires that the culture from which mRNA was extracted must be at a high population density to avoid the use of cumbersome volumes of culture. This requires a fully grown culture that is in the midst of bacteriocin production.

Induction often plays a very important role in the regulation of bacteriocin production. The operons of class II bacteriocins may include genes for the bacteriocin, an immunity peptide, transport machinery and a regulatory feedback system. Transcription of the bacteriocin structural gene is regulated by the induction factor, histidine kinase, response regulator, dedicated transporter, and transport accessory genes. A concentration-dependent signalling, or 'quorum sensing', extracellular concentration of the induction peptide determines whether a culture will produce bacteriocin (Kleerebezem *et al.*, 1997; Kleerebezem and Quadri, 2001; Quadri, 2002; Quadri, 2003). Once the concentration of the induction peptide reaches a critical level, the histidine kinase is activated and phosphorylates the response regulator, which has a regulatory effect on transcription of the target operon (Dutta *et al.*, 1999; Kim and Forst, 2001; Stephenson and Hoch, 2002). This upregulates transcription of the bacteriocin structural and immunity genes, which results in increased translation of the corresponding mRNA (Varughese, 2002). The transport machinery processes the bacteriocin to its mature form and exports it from the cell, where the bacteriocin becomes active in the extracellular environment.

The production of piscicolin 126 is regulated in a manner such that presence of the induction peptide in the culture supernatant induces production of piscicolin 126 in *C. maltaromaticum* UAL26. Because the genes for carnobacteriocin BM1 and its immunity peptide are also present in the *C. maltaromaticum* UAL26 genome, induction of carnobacteriocin BM1 production could be linked to induction and control of the piscicolin 126 operon. The objectives of this study were to isolate and compare the amount of mRNA transcribed for genes in the piscicolin 126 and carnobacteriocin BM1 operons in *C. maltaromaticum* UAL26 and JG126 at different growth temperatures, to evaluate the effect of induction of the piscicolin 126 operon by the piscicolin 126 induction peptide, and to determine its role in control of bacteriocin expression and production.

5.2. Materials and methods

5.2.1. Bacterial strains and growth media

C. maltaromaticum UAL26, LV17, LV17A, LV17B, and LV17C were acquired from the University of Alberta Lactic Acid Bacteria collection (University of Alberta, Edmonton, AB). *C. maltaromaticum* JG126 was acquired from the Australian Food Industry Science Centre Culture Collection (Werribee, Victoria, Australia).

Stock cultures of all *C. maltaromaticum* strains were maintained at -70°C in All Purpose Tween broth (APT; Difco; BD Diagnostic, Sparks, MD) with glycerol (20% vol./vol.; Fisher Scientific, Edmonton, AB). Cultures of *C. maltaromaticum* used in this study were prepared by inoculation of cells from frozen stock culture into APT broth at 15°C or 25°C. Cultures were subcultured at least once more at 1% vol./vol. before use in experiments.

Listeria monocytogenes ATCC 43256 was grown from frozen stock culture in APT broth at 37°C for 24 h, subcultured at least once before use in experiments, and used as the indicator organism to determine arbitrary activity units (A.U.) by the spot-on-lawn assay. Arbitrary activity units are calculated as the reciprocal of the highest dilution in a doubling dilution series that showed a clear zone of inhibition, and are expressed in

A.U./ml (Ahn and Stiles, 1990). For the spot-on-lawn assay, 10 µl of each dilution of cell-free (centrifuged at 10,000 x *g* for 5 min) heated (65°C for 30 min) supernatant was spotted onto APT agar plates, and overlayered with 7 ml of soft APT agar (0.75% agar) seeded with a 1% inoculum of *L. monocytogenes* ATCC 43256. Plates were incubated at 37°C for 24 h, zones of inhibition were measured, and A.U./ml calculated.

Antibacterial activity produced by *C. maltaromaticum* LV17, LV17A, or LV17B was assayed using *C. maltaromaticum* LV17C as the indicator organism.

5.2.2. Bacteriocin production by *C. maltaromaticum* UAL26 and JG126

To determine the optimum time for RNA isolation, *C. maltaromaticum* UAL26 and *C. maltaromaticum* JG 126 were grown in APT broth from frozen stock at 15°C and 25°C for 24 h. These cultures were subcultured a second time at an inoculum volume of 10%, and grown at 15 and 25°C for 24 h. The cultures were subcultured a third time using an inoculum volume of 1% and grown for 24 h. The cultures (10 ml) were centrifuged at 13,000 x *g* for 10 min. All but 100 µl of the supernatant was removed, and the pelleted cells were resuspended in the remaining volume. This was used to inoculate 10 ml of fresh APT broth. Samples for the spot-on-lawn assay were removed every 10 min for cultures incubated at 25°C, and every 20 min for those incubated at 15°C. The samples were measured for bacteriocin titre by the spot-on-lawn assay.

5.2.3. Synthesis, purification, and analysis of the piscicolin 126 induction peptide

The piscicolin 126 induction peptide was synthesized using fluorenylmethoxycarbonyl methodology (Bodanszky, 1993) and characterized (Figures 6.2, 6.3, and 6.4) by our collaborators, Darren J. Derksen and Dr. John C. Vederas (Department of Chemistry, University of Alberta). The synthetic peptide sequence was based on the translated amino acid sequence from the piscicolin 126 operon in *C. maltaromaticum* JG126 (Jack *et al.*, 1996). To ensure that the synthetic peptide was pure and of the correct molecular mass, it was subjected to RP-HPLC and MALDI-TOF MS analyses by our collaborators.

The purified peptide (0.239 mg) was diluted with 91 µl of sterile water and frozen (-20°C) for further use. The synthetic peptide solution (1 mM) was diluted to 10 µM concentration (10 µl in 1 ml APT broth) and heated at 70°C for 30 min, then cooled at 4°C for 10 min.

5.2.4. Induction of bacteriocin production in *C. maltaromaticum* UAL26, JG126, and LV17

To determine if bacteriocin production could be induced using spent supernatant, *C. maltaromaticum* UAL26, JG126, LV17, LV17A, LV17B, and LV17C were grown in 10 ml of APT broth at 15°C for 24 h and subcultured three times. Each culture was assayed by the spot-on-lawn assay to determine the bacteriocin titre. The cultures were centrifuged at 13,000 x *g* for 10 min. The supernatant of each culture was decanted into 14 ml plastic tubes. Half of the supernatant was removed and filtered through a 10 µm low-protein binding PVDF Millex GV Durapore syringe filter (Millipore, Cork, Ireland). Supernatant (5 ml) was filtered into a fresh 14 ml plastic tube and stored at 4°C for further use. The remaining supernatant (5 ml) was heated at 70°C for 30 min, then stored at 4°C for further use in experiments.

Each sample of spent supernatant was assayed for bacteriocin activity at 1% concentration in sterile APT broth by the spot-on-lawn assay. This was to ensure there was no background bacteriocin activity at a 1% dilution that could affect the spot-on-lawn results of an induced culture. *C. maltaromaticum* UAL26 cultures grown at 25°C were induced by addition of 1% vol./vol. of spent supernatant at the time of subculturing.

To determine the effect of synthetic induction peptide concentration on bacteriocin production in *C. maltaromaticum* UAL26 grown at 25°C, the induction peptide was serially diluted into APT broth to obtain concentrations of 10 μM to 10 pM. The APT broth containing induction peptide was heated at 70°C for 30 min, cooled to 25°C, and inoculated with 1% vol./vol. *C. maltaromaticum* UAL26 culture previously grown at 25°C. The cultures were incubated at 25°C for 24 h and then tested for bacteriocin activity by spot-on-lawn assay.

To determine if induction of bacteriocin production could be diluted out by subculturing, *C. maltaromaticum* UAL26 was grown from frozen culture in 10 ml APT broth at 15°C for two subcultures, and then subcultured a third time by serial dilution to a concentration of 10^{-10}. Likewise, *C. maltaromaticum* JG126 was grown at 25°C and serially diluted in the same manner. The cultures were incubated for up to 4 d, and tested for bacteriocin activity by the spot-on-lawn assay. The cultures that grew but did not exhibit bacteriocin activity were subcultured again (using the 10^{-10} culture) to confirm the absence of bacteriocin production by the growing cultures. These bacteriocin-negative cultures were subcultured into APT broth with 10 μM of piscicolin 126 induction peptide and tested for bacteriocin activity using the spot-on-lawn assay.

To evaluate DNA and protein sequence similarity between the induction peptide genes, comparisons of the sequences of the induction genes from *C. maltaromaticum* LV17B (Stiles, 1995; Quadri *et al.*, 1997) and *C. maltaromaticum* JG126 (Jack *et al.*, 1996; Wettenhall *et al.*, 2000) were made using the DNAStar program (DNAStar Inc., Madison, WI) using the ClustalW alignment method (Slow/Accurate, IUB settings for DNA sequences, Slow/Accurate, Gonnet settings for translated protein sequences).

5.2.5. Preparation of total RNA samples

All RNA work was carried out wearing gloves, and taking precautions to prevent RNase contamination. Subcultures used in the preceding experiment were used for isolation of total RNA. When the optimum time for isolation of bacteriocin structural mRNA (defined as the time when ~100 to 200 A.U./ml had been reached in the spot-on-lawn assays), 10 ml of each culture was centrifuged at 13,000 x *g* for 10 min. The supernatant was discarded except for 500 μl, which was heated in a water bath at 65°C for 1 h, and then used in a spot-on-lawn assay to determine the bacteriocin titre at the time of mRNA isolation. The pelleted cells were resuspended by vortex in 1 ml RNApro Solution (FastRNA Pro Blue Kit; Qbiogene, Carlsbad, CA) and the cell suspension was transferred into a sterile 2 ml bead-beating tube (Qbiogene) containing Lysing matrix B (Qbiogene). The samples were shaken for 3 min in a Mini Beadbeater (Biospec Products, Bartlesville, OK) set at maximum speed. The tubes were cooled on ice for 5 min to allow beads to settle and centrifuged at 15,000 x *g* for 5 min. The upper phases of the samples were transferred to microcentrifuge tubes and incubated at 25°C for 5 min. Chloroform (300 μl; Fisher Scientific) was added and the samples mixed by vortexing for 10 s. The samples were incubated at 25°C for 5 min and centrifuged at 15,000 x *g* at 4°C for 5 min. The aqueous phases were transferred to new microcentrifuge tubes and 500 μl of cold (4°C) 100% ethanol was added to each sample. The tubes were inverted five times to mix the contents and incubated at -20°C for 30 min to precipitate the RNA. The precipitated samples were centrifuged at 15,000 x *g* at 4°C for 15 min. The supernatant was removed and the RNA pellet was washed with 500 µl of cold (4°C) 75% ethanol [prepared using diethylpyrocarbonate(DEPC)-treated RNase-free water]. The wash solution was removed and the RNA was dried at 25°C for 5 min. DEPC-treated RNase-free water (100 µl) was used to resuspend the RNA pellets, which were stored at -80°C.

To determine the effect of the synthetic induction peptide on bacteriocin production, *C. maltaromaticum* UAL26 and JG126 were each grown in 10 ml of APT broth, and *C. maltaromaticum* UAL26 was grown in APT broth supplemented with 5 µM induction peptide at 25°C for 24 h. The cultures were subcultured twice in APT broth at an inoculum volume of 1%. The mRNA from these cultures was isolated 40 min after the final subculture as described above.

5.2.6. Northern analysis of the RNA samples

The concentration of RNA was quantified using a GeneQuant spectrophotometer (Biochrom Ltd., Cambridge, U.K.) to measure absorbance at 260 nm. The GeneQuant also compared the ratio of A_{260} to A_{280}. Final RNA concentration in µg/ml was calculated as the multiple of the A_{260} x sample dilution x 40 µg/ml.

All glassware and utensils used in experiments involving RNA were heated at 250°C for 8 h. Ultra-pure agarose (1.4 g; Invitrogen Canada Inc., Burlington, ON) was added to 120 ml of sterile DEPC-treated RNase free water (Qbiogene) and brought to a boil in a microwave oven. The solution was cooled to approximately 55°C and 14 ml of 10 x MOPS [3-(N-morpholino)propanesulphonic acid, 10 mM ethylenediaminetetraacetic acid (EDTA); BioShop Canada, Inc., Burlington, ON)] buffer was added. Formaldehyde (7.6 ml) was added as a 37% solution, and the solution was mixed gently by shaking. The agarose gel was poured into an agarose gel electrophoresis unit and allowed to solidify for 1 h, then immersed in 1 x MOPS buffer. RNA samples were prepared by mixing 15 µg of total cellular RNA extract in fresh sample buffer [12 samples: 96 µl of deionized formamide (Invitrogen) + 34.6 µl of 37% formaldehyde + 69.4 µl of loading dye {40 µl of 10 x MOPS + 46 µl of DEPC-treated RNase-free water + 6 µl of ethidium bromide (10 mg/ml) + 20 µl of glycerol + 20 µl of saturated bromophenol blue solution}]. The mixture was heated at 100°C for 2 min and immediately cooled on ice. The tubes were centrifuged at 10,000 x *g* for 5 s to collect the condensate, then the samples were loaded onto the agarose gel.

The agarose gel was run at 100 V until the bromophenol blue dye had reached the lower portion of the gel (approximately 4.5 h). The gel was analysed on a Gel Doc 1000 UV transilluminator with the Molecular Analyst v. 2.12 program (BioRad Laboratories, Hercules, CA). The gel was prepared for Northern transfer by soaking it in 2 changes of 10 x SSC buffer (87.65 g of NaCl + 44.1 g of trisodium citrate/L, pH 7.0) for 10 min each. The Northern transfer was done for 18 h by stacking NitroPure supported 0.45 micron pure nitrocellulose membrane (Micron Separations Inc., Westborough, MA), Whatman filter paper (Whatman International, Inc., Kent, U.K.), and paper towels on top of the gel. After the transfer was completed, the membrane was visualized by UV illumination. The membrane was allowed to dry in ambient air at 25°C for 1 h, and then the RNA was fixed to the membrane by heating in a vacuum oven at 80°C for 2 h.

DNA probes for hybridization to the fixed membrane were prepared using the following primers:

piscicolin 126:

primer LG126F2: 5'-ATATGAATTCATGAAAACTGTTAAAGAACT-3', and

primer LG126R2: 5'-ATATTCTAGATTATCCTTTGTTCCAACC-3'.

carnobacteriocin BM1:

primer BM1F1: 5'-ATATGAATTCATGAAAAGCGTTAAAGAACT-3', and

primer BM1R1: 5'-ATATTCTAGATTAATGTCCCATTCCTGC-3'.

control 16S rRNA:

primer RNA3: 5'-TAGCGGTGAAATGCGT- 3', and

primer RNA4: 5'-TCGAATTAAACCACATGCTC- 3'.

These primers were chosen because they spanned the majority of the sequences transcribed in production of mRNA for the genes to be assayed. The control 16S rRNA primers were designed based on the *C. maltaromaticum* 16S ribosomal RNA sequence.

For all reactions, DNA was amplified in 100 µl PCR reactions using 0.5 µl of Platinum Taq DNA polymerase High Fidelity (Invitrogen), 10 µl of 10 x HIFI Platinum Taq Buffer, 4 µl [illegible] 50 mM MgSO$_4$, 1 µl of each primer at 200 nM (pairings of the primers listed above), 2 µl of 10 mM dNTP solutions (Invitrogen), and 1 µl of purified DNA template isolated from *C. maltaromaticum* UAL26.

The following PCR protocol was used on a GeneAmp PCR system 2400 thermal cycler (version 2.11; Perkin Elmer Corp., Norwalk, CT): 5 min denaturation at 94°C, 40 cycles of: 1 min denaturation at 94°C, 1 min annealing at 50°C, 1 min extension at 68°C, a final 5 min extension at 72°C, and the samples were cooled and held at 4°C. The samples were cleaned using a QiaQuick PCR purification column (Qiagen Inc., Hilden, Germany) and eluted in 30 µl of DEPC-treated RNase free water. Each reaction mixture

(5 μl) was loaded onto a 1% w./vol. agarose (BD Diagnostics) gel [dissolved in 1 x TBE buffer: 10.778 g of tris(hydroxymethyl)aminomethane (Tris), 0.744 g of disodium ethylenediaminetetraacetic acid (EDTA), 5.5 g of boric acid/L] and separated in 1 x TBE buffer by electrophoresis at 110 V for approximately 30 min using a Mini-Sub Cell GT gel box (BioRad). The gel was visualized with a Gel Doc 1000 UV transilluminator (BioRad) and photographed.

The remainder of the PCR product sample was labelled using a Random Primers DNA Labelling system (Invitrogen). For each sample, the following reaction was set up: each PCR product (23 μl) was placed in a microcentrifuge tube, heated at 100°C for 5 min, and held on ice. To each tube 15 μl of random primers buffer mixture, 2 μl of dATP, 2 μl of dGTP, 2 μl of dTTP, and 5 μl of [α-^{32}P]dCTP, 3000 Ci/mmol, 10 μCi/μl (Amersham Biosciences Corp., Piscataway, NJ) were added. The mixture was briefly mixed by vortexing, 1 μl of Klenow fragment (Invitrogen) was added, the tube was mixed by vortexing, and centrifuged at 10,000 x *g* for 5 s. The tubes were incubated at 25°C for 1 h, and 5 μl of stop buffer (Invitrogen) was added to stop the reaction.

Prior to hybridization, the RNA membrane was cut into three equal pieces, with each piece containing a set of the four mRNA samples. The membrane was submerged in deionized water for 5 s to remove excess salt. The membrane pieces were placed into glass hybridization tubes (5 cm x 20 cm). Prehybridization solution [8 ml, 6 x SSPE (900 mM sodium chloride, 60 mM monosodium phosphate, 60 mM EDTA), 0.5% SDS, and 5 x Denhardt's solution {50 x Denhardt's solution = 1% Ficoll 400 (Serva Electrophoresis Inc., Heidelberg, Germany), 1% polyvinylpyrrolidone (Sigma-Aldrich Canada, Oakville, ON), 1% bovine serum albumin fraction IV (Sigma-Aldrich)}] was warmed to 60°C, added to each membrane, and prehybridized in a hybridization oven (series 942; Labtime Instruments Inc., Melrose Park, IL) at 65°C for 2 h. Hybridization was done by adding 100 µl of tRNA (10 mg/ml) to each labelled probe (50 µl), heating the mixture at 100°C for 2 min, and cooling on ice. This solution was added to the prewarmed prehybridization solution in the hybridization tubes containing the membrane pieces. Hybridization was done in a hybridization oven at 65°C for 24 h.

The hybridized membrane was washed 3 times with 2 x SSPE containing 0.1% SDS at 25°C for 10 min, and then once in 0.1 x SSC containing 0.1% SDS at 65°C for 10 min. The hybridized membranes were sealed in a plastic bag and exposed to a BAS-IP-SR 20 x 25 cm imaging plate (Fuji Photo Film Co. Ltd., Valhalla, NY) in a BAS 20 x 25 cm cassette (FujiFilm) for 3 h. The image was visualized and recorded using a BAS-1800 IP reader running the Image Reader 1.7E program (FujiFilm).

5.2.7. RT-PCR of the RNA samples

Reverse transcription PCR was performed twice: on the four RNA samples used in the Northern Transfer described in section 5.2.6, and on the three RNA samples from the cultures with induction peptide added. RNA samples were treated with DNase (DNA-free; Ambion, Austin, TX) to ensure that residual DNA would not act as a template during PCR. To remove DNA from the four purified RNA samples, purified total cellular RNA (17 µl) was added to 2 µl DNase I buffer in a microcentrifuge tube, and mixed by vortexing. Recombinant DNase (1 µl) was added, the mixture was mixed by vortexing, and incubated at 37°C for 1 h. DNase inactivation reagent (2 µl) was added to the reaction mixture. Inactivation was done at 25°C for 3 min, with gentle mixing every 30 s to ensure suspension of the inactivation reagent. The tubes were centrifuged at 10,000 x *g* for 1.5 min and the supernatant was transferred to a new microcentrifuge tube.

The following primers were used:

pisI: *primer FI: 5'-CTAATATCCATATCTAATATTG-3',
primer RI: 5'-ATGGGTAAGTTAAAATGGTTT-3',
pisA: primer LG126F2: 5'-ATATGAATTCATGAAAACTGTTAAAGAACT-3',
*primer LG126R2: 5'-ATATTCTAGATTATCCTTTGTTCCAACC-3',
pisN: primer FN: 5'-ATGAACGATAAAAAATACTTG-3',
*primer RN: 5'-TTAGCATTTTTTAAAAAAAGAAA-3',
pisK: primer FK1: 5'-TTGTAAAGTATAGTTCTATTA-3',
*primer RK1: 5'-ATGTTTAAATTTATTTAGCTTTT-3',
pisR: primer FR: 5'-GAAGATCAAATCATTCAAC-3',
*primer RR: 5'-TTTGAACTAACCTTTGCCG-3',
pisT: primer FT1: 5'-GGTGTTAGTGGATCTGGA-3',
*primer RT1: 5'-CTTCAATAAAGCACGAGCA-3',
pisE: primer FE: 5'-ATGCATAATAATAACTGGCG-3',
*primer RE: 5'-AGTGGTACTTGTAGTTTAT-3',
cbnBM1: primer BM1F1: 5'-ATATGAATTCATGAAAAGCGTTAAAGAACT-3',
*primer BM1R1: 5'-ATATTCTAGATTAATGTCCCATTCCTGC-3',
control 16S rRNA: primer RNA3 5'-TAGCGGTGAAATGCGT- 3',
*primer RNA4: 5'-TCGAATTAAACCACATGCTC- 3'.

* = antisense primer

For each RNA sample for each gene tested, 1 μl of each antisense (noncoding strand) gene specific primer, 1 μl of dNTP (10 μM) mixture, and a volume of RNA containing 2 μg of total RNA sample, was added to each tube and DEPC-treated RNase free sterile water was added to bring the reaction volume to 13 μl. The reaction mixtures were heated at 65°C for 5 min and cooled on ice for 1 min. The tubes were centrifuged at 10,000 x *g* for 5 s, and 4 μl of 5 x first-strand buffer, and 1 μl of 0.1 M dithiothreitol (DTT) were added to each tube. The tubes were vortexed briefly and incubated at 42°C for 2 min. Superscript III reverse transcriptase (1 μl, 200 U/μl; Invitrogen) was added. The reactions were mixed by vortexing, and incubated at 50°C for 1 h. The reaction was inactivated by heating at 70°C for 15 min. Residual RNA was removed by treating the mixtures with RNase A (0.5 μl; 2 mg/ml) and incubating at 37°C for 30 min.

PCR was done according to the protocol described in section 5.2.6, using DNA template from the reverse transcription reactions and amplifying for 25 cycles. The samples (15 μl) were analysed by gel electrophoresis as described in section 5.2.6.

5.2.8. Construction of the plasmid pLG26PISNC and transformation of *C. maltaromaticum* UAL26

To determine if the induction peptide of piscicolin 126 could induce bacteriocin production *in vivo*, it was necessary to transform *C. maltaromaticum* UAL26 with a plasmid construct containing the gene for the PisN induction peptide. To construct the plasmid, DNA primers (Applied Biosystems 394 DNA/RNA Synthesizer, DNA Core Facility, University of Alberta) were designed based on the sequence of the piscicolin 126 induction gene from *C. maltaromaticum* JG126 (Jack *et al.*, 1996; Gibbs *et al.*, 2000), with a 10 bp sequence at the beginning (5'-end) to allow the appropriate restriction endonucleases to cut the DNA fragment amplified by PCR. The primers were used in a PCR reaction (Sambrook and Russell, 2001) to amplify the piscicolin 126 induction gene. The primers used to amplify the fragment were designed to contain the *Cla*I and *Sph*I restriction sites at either end of the amplified region. The sequence of the primers was: primer PISNCF1: 5'-AGAGATCGATAATTGGAGGGAATAAAAATGAACG-3', and primer PISNCR2: 5'-ATATGCATGCTCATTAGCATTTTTAAAAAAAGAAAAA-3'.

DNA was amplified in a 100 μl PCR reaction using 0.5 μl of Platinum Taq DNA polymerase High Fidelity (Invitrogen), 10 μl of HIFI Platinum Taq Buffer, 4 μl of 50 mM $MgSO_4$, 1 μl of each primer at 200 nM (pairings of the primers listed above), 2 μl of 10 mM dNTP solutions (Invitrogen), and 1 μl of purified DNA template isolated from *C. maltaromaticum* UAL26.

The following PCR protocol was used: 2 min denaturation at 94°C, 40 cycles of: 1 min denaturation at 94°C, 1 min annealing at 60°C, 2 min extension at 68°C, then a final 5 min extension at 72°C, and the sample was cooled and held at 4°C. The reaction mixture (10 μl) was loaded onto a 1% w./vol. agarose gel, separated by electrophoresis, and visualized as described in section 5.2.6.

To ligate the *pisN* PCR product into the vector for transformation of *C. maltaromaticum* UAL26, the PCR amplified fragment was cleaned using a QiaQuick PCR purification column, eluted with a final volume of 50 μl EB buffer (10 mM TrisCl, pH 8.5), and double digested with the appropriate restriction endonucleases (*Cla*I and *Sph*I in 10 x React 1, each 10% vol./vol.). Plasmid vector pMG36e (Van de Guchte *et al.*, 1989) was cut using the same protocol, without the QiaQuick purification step. The fragment and plasmid were incubated at 37°C for 2 h and purified by agarose gel electrophoresis using a gel extraction kit (Qiagen) according to the manufacturer's protocol. Ligation was done by mixing the cut fragment with the cut plasmid, and adding 10 μl of 5 x ligation buffer and 5 μl of T4 DNA ligase enzyme (Invitrogen). The solution was mixed by vortexing and incubated at 25°C for 2 h. The resulting plasmid was named pLG26PISNC.

To transform *C. maltaromaticum* UAL26 with pLG26PISNC, the ligation mixture containing pLG26PISNC, a 100 μl tube of electrocompetent *C. maltaromaticum* UAL26, and an electroporation cuvette were placed on ice. Plasmid DNA (10 μl) was added to the electrocompetent cells, mixed briefly, and transferred into the electroporation cuvette. The mixture was held on ice for 2 min, and electroporated using a Gene Pulser Electroporator (BioRad) at 2.5 kV and 200 Ω resistance. APT supplemented with 0.5 M sucrose and 20 mM $MgCl_2$ (1 ml) was added to the cuvette and the mixture transferred to a microcentrifuge tube. The culture was incubated at 25°C for 4 h, plated in 100 μl volumes onto APT agar plates containing erythromycin (5 μg/ml; Fisher Scientific) and plates were incubated at 25°C. Colonies that grew within 6 d were picked from the agar plates and grown in APT broth with erythromycin (5 μg/ml) at 25°C for 24 h. To determine if the transformant cultures carried pLG26PISNC, cultures (1 ml) were centrifuged at 20,000 x *g* for 2 min, the supernatant discarded, and the cells were resuspended in 250 μl ice-cold STE (10 mM TrisCl, 0.1 M NaCl, and 1 mM EDTA, pH 8) buffer. The centrifugation was repeated, the cells were lysed by the alkaline lysis method (Sambrook and Russell, 2001), and extracted by vortexing for 10 s with 500 μl of buffer-saturated phenol. This mixture was separated by centrifuging at 10,000 x *g* for 1 min, and the aqueous layer was transferred to a new microcentrifuge tube. An equal volume of chloroform:isoamyl alcohol (24:1) was added to the tube, and separated by centrifuging at 10,000 x *g* for 1 min. The aqueous layer was transferred to a new microcentrifuge tube. Purified pLG26PISNC was cut with the appropriate restriction enzymes for 1 h as described above, and loaded onto a 1% w./vol. agarose gel, separated by electrophoresis, and visualized as described in section 5.2.6.

5.3. Results

5.3.1. Bacteriocin production by *C. maltaromaticum* UAL26 and JG126

The time of bacteriocin production during growth of cultures stored at 15°C and 25°C is shown in Figure 5.1. Using the spot-on-lawn assay, it was observed that bacteriocin-producing cultures of *C. maltaromaticum* UAL26 or *C. maltaromaticum* JG126 subcultured at an inoculum of 1% vol./vol. would produce sufficient bacteriocin activity for detection using a spot-on-lawn assay within 2 h. Even though the cultures had been diluted to 1% of their maximum population (diluting out any residual bacteriocin activity from the inoculum volume), the bacteriocin titre increased rapidly until it had reached that of a fully grown culture. This enabled the determination of the time during culture growth at which the mRNA of the bacteriocin structural gene should be transcribed at its highest level. Using a full population of cells (~10^9 CFU/ml) allowed greater amounts of the desired mRNA to be harvested during this time. The times that RNA was harvested from the cultures grown at 15°C or 25°C are shown in Figure 5.1. Because *C. maltaromaticum* JG126 grown at 15°C produced bacteriocin at a faster rate than *C. maltaromaticum* UAL26 grown at 15°C, RNA was harvested from *C. maltaromaticum* JG126 after 80 min, and from *C. maltaromaticum* UAL26 after 120 min.

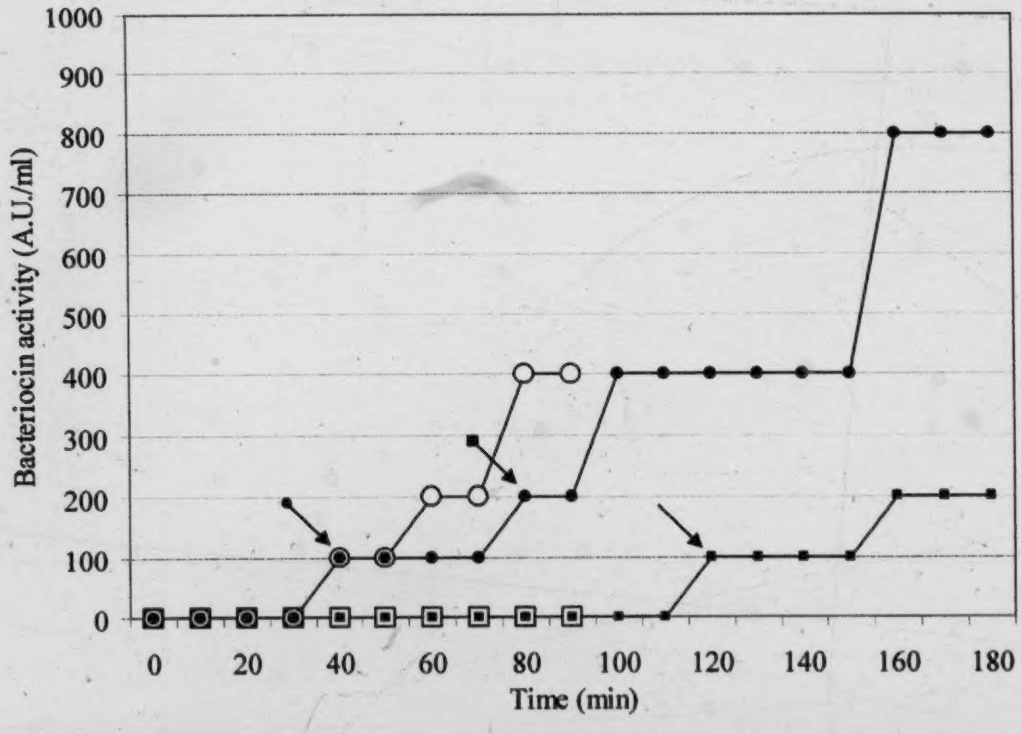
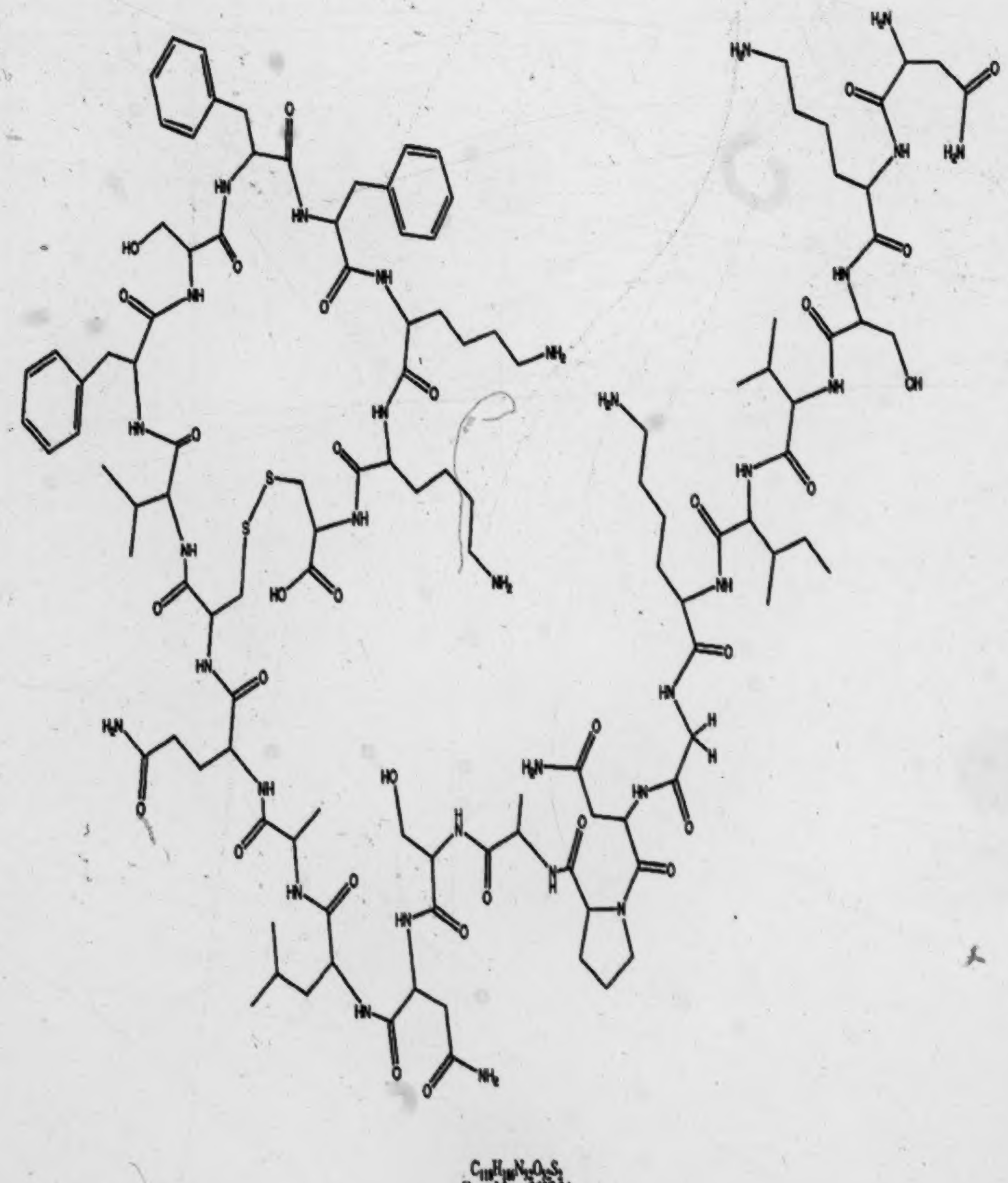


Figure 5.1. Bacteriocin production by *C. maltaromaticum* UAL26 grown at 15°C (▪) and 25°C (☐), and *C. maltaromaticum* JG126 grown at 15°C (●) and 25°C (○). Cultures were prepared by inoculating APT broth with 10^9 CFU/ml. *L. monocytogenes* ATCC 43256 was used as the indicator organism. Arrows indicate time of mRNA isolation: ●→: *C. maltaromaticum* UAL26 and *C. maltaromaticum* JG126 grown at 25°C; ◆→: *C. maltaromaticum* JG126 grown at 15°C culture; →: *C. maltaromaticum* UAL26 grown at 15°C.

5.3.2. Synthesis, purification, and analysis of the piscicolin 126 induction peptide

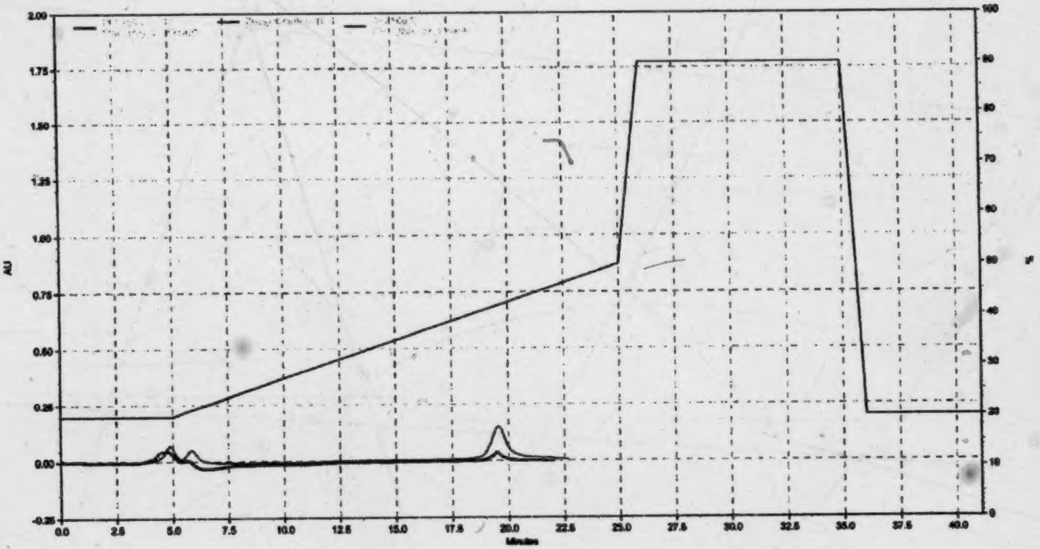
The structure of the synthetic induction peptide is shown in Figure 5.2. It was synthesized and purified by RP-HPLC (Figure 5.3). The peptide was subjected to MALDI-TOF MS to determine its molecular mass. The MALDI-TOF MS results (Figure 5.4) had a peak at 2629.2 Da, which agreed with the calculated molecular weight of 2629.06 Da.



$C_{118}H_{186}N_{32}O_{32}S_2$
Exact Mass: 2627.34
Mol. Wt.: 2629.06

Figure 5.2. Diagrammatic structure of the piscicolin 126 induction peptide. Obtained from Darren Derksen (Department of Chemistry, University of Alberta).

A.)



B.)

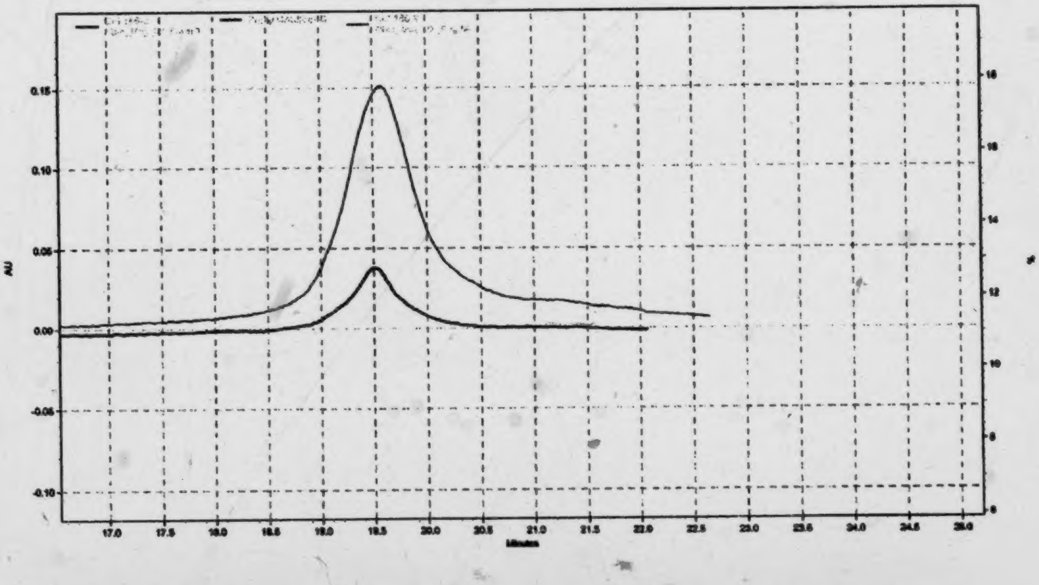


Figure 5.3. RP-HPLC chromatogram of purified synthetic piscicolin 126 induction peptide. A.) piscicolin 126 induction peptide solvent gradient and elution profile. B.) Expansion of the synthetic piscicolin 126 induction peptide peak. Obtained from Darren Derksen (Department of Chemistry, University of Alberta).

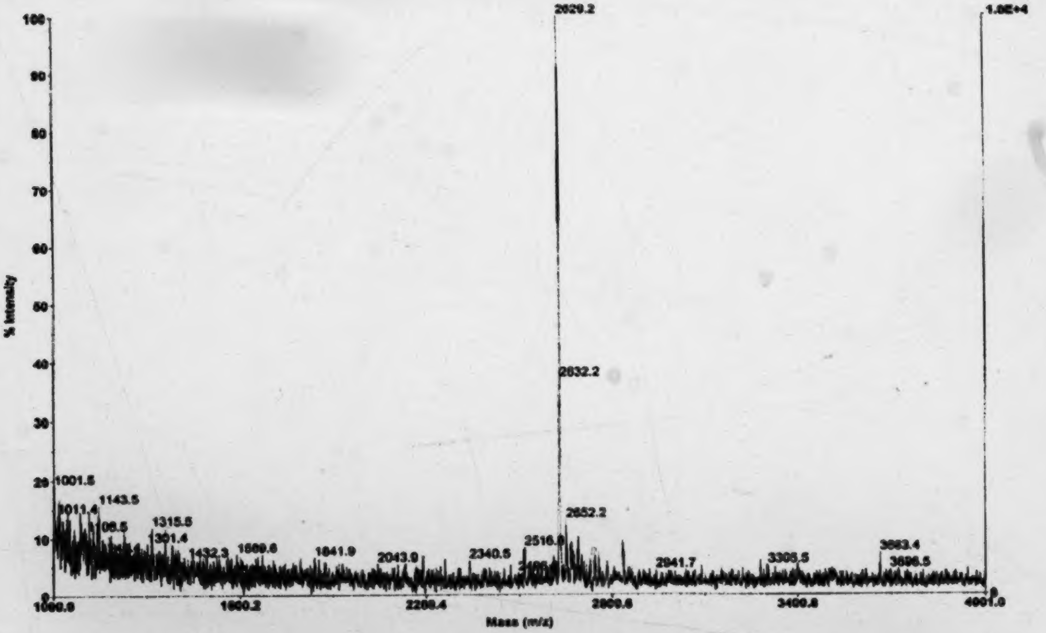


Figure 5.4. MALDI-TOF MS analysis of purified cyclized synthetic piscicolin 126 induction peptide. Obtained from Darren Derksen (Department of Chemistry, University of Alberta).

5.3.3. Induction of bacteriocin production in *C. maltaromaticum* UAL26, JG126, and LV17

C. maltaromaticum UAL26 grown at 25°C did not produce a detectable amount of bacteriocin activity, but *C. maltaromaticum* UAL26 grown at 15°C and *C. maltaromaticum* JG126 grown at 25°C produced 800 A.U./ml. Bacteriocin activity was not detected in 1% dilutions of the supernatant from *C. maltaromaticum* UAL26 grown at 15°C and *C. maltaromaticum* JG126 grown at 25°C. A 10 μM solution of the induction peptide (IP) in APT broth showed no antibacterial activity. Bacteriocin production could be induced in *C. maltaromaticum* UAL26 grown at 25°C by the addition of synthetic induction peptide, or 1% spent supernatant from either *C. maltaromaticum* UAL26 grown at 15°C or *C. maltaromaticum* JG126 grown at 25°C (Figure 5.5). The bacteriocin titre

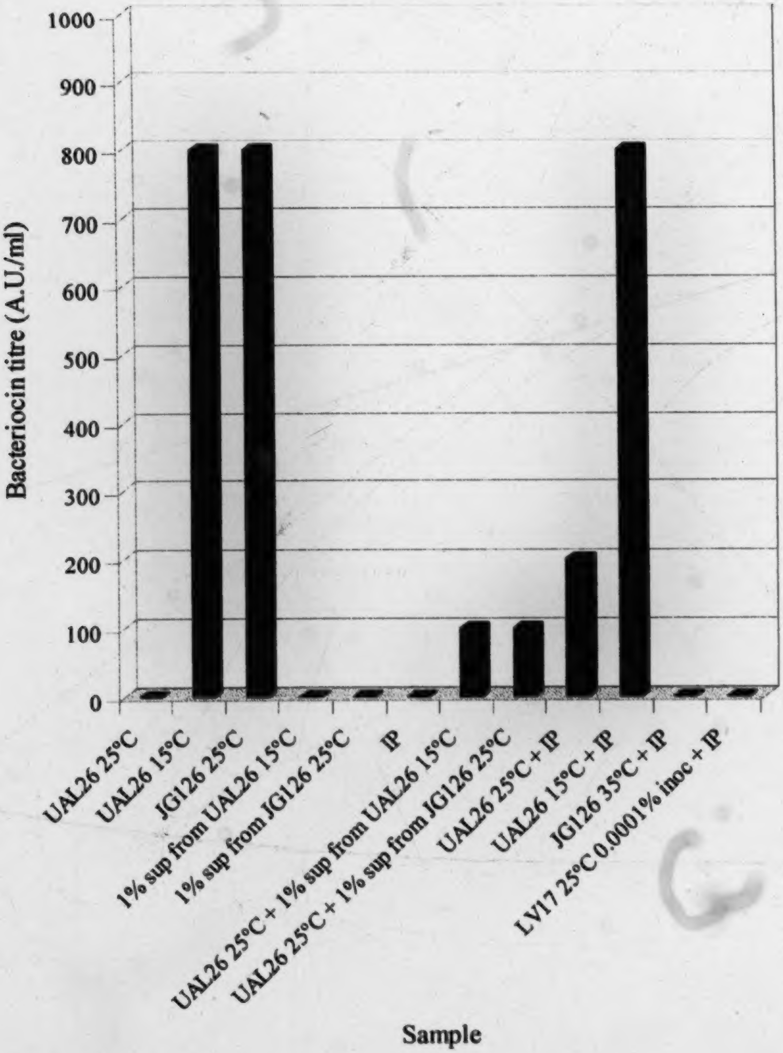


Figure 5.5. Bacteriocin activity of cultures of *C. maltaromaticum* UAL26 and *C. maltaromaticum* JG126 grown at 25°C, *C. maltaromaticum* JG126 grown at 35°C, and *C. maltaromaticum* LV17 grown at 25°C, supplemented with spent supernatant (sup) from *C. maltaromaticum* UAL26 grown at 15°C or *C. maltaromaticum* JG126 grown at 25°C or with 10 μM purified piscicolin 126 induction peptide (IP). (n=2)

reached a maximum of 100 A.U./ml when 1% spent supernatant was added, and reached 200 A.U./ml after three subcultures when the synthetic induction peptide was added. A subculture of *C. maltaromaticum* UAL26 from frozen stock supplemented with induction peptide and grown at 15°C reached 800 A.U./ml without the necessary time (5 d) to adapt to cold temperatures as previously observed (Chapter 2, this thesis). Heating at 70°C for 30 min or filtration of the spent supernatant did not affect the bacteriocin titre observed in induced cultures of *C. maltaromaticum* UAL26.

Cultures of *C. maltaromaticum* UAL26 supplemented with spent supernatant from *C. maltaromaticum* LV17 and LV17A took 48 and 24 h to grow, respectively, in comparison to 16 h for the other cultures. The supernatant from *C. maltaromaticum* LV17 or its derivatives did not induce bacteriocin production in *C. maltaromaticum* UAL26 grown at 25°C (data not shown).

C. maltaromaticum JG126 grew in 24 h when incubated at 35°C, and showed marked reduction in bacteriocin titre after the first subculture (100 A.U./ml), and no bacteriocin activity was detected after the second subculture (data not shown). As previously demonstrated (Franz *et al.*, 2000), *C. maltaromaticum* LV17 produces bacteriocin activity against *C. maltaromaticum* LV17C when subcultured at 1% inoculum, but bacteriocin activity was not detected when the inoculum size was reduced to 0.0001%. In the current study, bacteriocin-negative cultures of *C. maltaromaticum* JG126 and LV17 were grown in APT broth containing 10 μM piscicolin 126 induction peptide, but neither was induced to produce detectable amounts of bacteriocin activity.

Although the induction genes from *C. maltaromaticum* UAL26 and LV17B share sequence identity of ~60%, they are less similar (~45%) when aligned as peptides (Figure 5.6). The leader sequence shares higher identity in DNA (~60%) and amino acid (~40%) sequence, while the remainder of the induction gene (encoding the mature induction

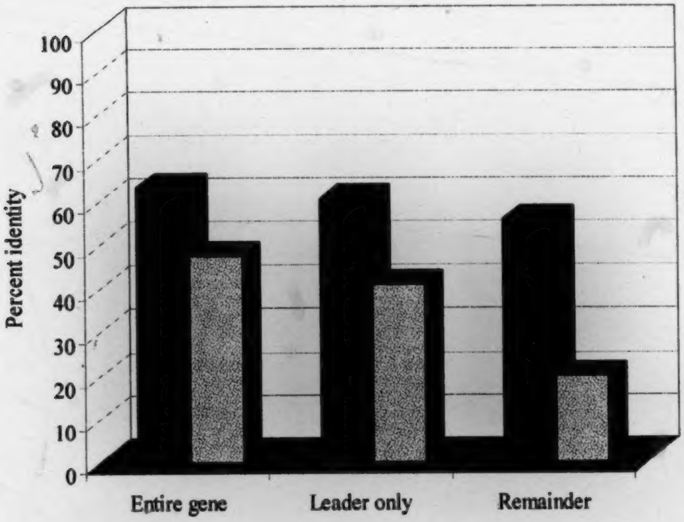


Figure 5.6. Percentage identity of the sequences of the induction gene and translated peptide of piscicolin 126 from *C. maltaromaticum* JG126 and carnobacteriocin B2 from *C. maltaromaticum* LV17B. DNA (■), protein (□).

peptide) shares almost as much identity in DNA sequence (~55%), yet much less identity when aligned as the mature peptide (~20%).

Addition of synthetic piscicolin 126 induction peptide at a concentration as low as 1 μM resulted in induction of bacteriocin production in *C. maltaromaticum* UAL26 grown at 25°C, but the presence of induction peptide at concentrations below 1 μM did not induce bacteriocin production (Table 5.1).

When cultures of *C. maltaromaticum* UAL26 grown at 15°C and *C. maltaromaticum* JG126 grown at 25°C are serially diluted to an inoculum below 10^{-5}, bacteriocin production is not induced (Table 5.2). Cultures do not regain the ability to produce bacteriocin upon further subculturing unless bacteriocin production is induced using synthetic piscicolin 126 induction peptide.

Table 5.1. Effect of concentration of synthetic piscicolin 126 induction peptide on bacteriocin production by *C. maltaromaticum* UAL26 grown at 25°C.

Concentration of induction peptide	Bacteriocin Activity (A.U./ml)
10 μM	200
1 μM	200
100 nmol	0
10 nmol	0
1 nmol	0
100 pmol	0
10 pmol	0

Table 5.2. Bacteriocin activity of serially diluted cultures of *C. maltaromaticum* UAL26 grown at 15°C and *C. maltaromaticum* JG126 grown at 25°C as detected by the spot-on-lawn assay using *L. monocytogenes* 43256 as the indicator organism. (n=2)

		Bacteriocin Activity	
Subculture	Inoculum level	*C. maltaromaticum* UAL26	*C. maltaromaticum* JG126
1	10^{-2}	+	+
2	10^{-2}	+	+
3	10^{-1}	+	+
	10^{-2}	+	+
	10^{-3}	+	+
	10^{-4}	+	+
	10^{-5}	+	+
	10^{-6}	-	-
	10^{-7}	-	-
	10^{-8}	-	-
	10^{-9}	-	-
	10^{-10}	-	-
4	10^{-2}	-	-
	10^{-4}	-	-
	10^{-6}	-	-
	10^{-8}	-	-
	10^{-10}	-	-
5	10^{-2}	-	-
	10^{-2} + IP	+	+

(+) bacteriocin activity detected, (-) bacteriocin activity not detected
IP: Induction peptide (10 μM) added

5.3.4. Northern analysis of the RNA samples

The concentrations of the RNA samples harvested from the cultures used to determine the effect of temperature on RNA transcription of the piscicolin 126 operon, and the effect of the presence of the synthetic induction peptide are shown in Table 5.3 and Table 5.4, respectively. The results indicate that a sufficient amount of RNA for Northern analysis and RT-PCR was isolated from all samples. The concentration of RNA isolated from *C. maltaromaticum* JG126 was higher than that isolated from *C. maltaromaticum* UAL26 regardless of growth temperature (Table 5.3).

Table 5.3. Concentration of total cellular RNA isolated from *C. maltaromaticum* UAL26 and JG126 grown at 15°C and 25°C.

Culture and growth temperature	RNA concentration (μg/μl)
UAL26 25°C	2.830
JG126 25°C	1.786
UAL26 15°C	3.488
JG126 15°C	1.948

Table 5.4. Concentration of total cellular RNA isolated from *C. maltaromaticum* UAL26, *C. maltaromaticum* UAL26 supplemented with synthetic induction peptide (IP), and *C. maltaromaticum* JG126 grown at 25°C.

Culture and growth temperature	RNA concentration (μg/μl)
UAL26 25°C	3.976
UAL26 25°C + IP	3.840
JG126 25°C	3.480

Northern analysis was done to compare the amount of piscicolin 126 and carnobacteriocin BM1 structural mRNA transcribed in *C. maltaromaticum* UAL26 and *C. maltaromaticum* JG126 grown at 15 and 25°C. The DNA probes used for labelling were created by PCR and were analysed to ensure enough product of the correct size had been synthesized. The PCR product bands (Figure 5.7) were of the expected molecular size based on the calculated length from the DNA sequence of the piscicolin 126 operon in *C. maltaromaticum* JG126.

Figure 5.7. PCR amplification of the DNA probes used for labelling and hybridization to the RNA transfer membrane. Lane 1: 100 bp marker; lane 2: piscicolin 126 probe; lane 3: carnobacteriocin BM1 probe; lane 4: 16S rRNA control probe.

The RNA samples were visualized after the completion of electrophoresis. The results showed the presence of the 23S and 16S ribosomal RNA subunits, and a smear of bands above and below the 23S and 16S bands (Figure 5.8). These smears indicate that the mRNA has not been degraded by RNase action. The presence of mRNA indicates that the samples were acceptable for Northern transfer and probe hybridization.

After hybridization, radioactivity from the labelled probe binding to the target mRNA was visualized (Figure 5.9). The autoradiography of the piscicolin 126 and carnobacteriocin BM1 probes to the RNA samples isolated from *C. maltaromaticum* UAL 26 and JG126 showed that the probe could bind to its target mRNA in samples isolated from *C. maltaromaticum* UAL 26 grown at 15°C and *C. maltaromaticum* JG126 grown at 15 and 25°C. The probes did not bind to their target mRNA in the sample isolated from *C. maltaromaticum* UAL 26 grown at 25°C, indicating that the bacteriocin structural genes were not transcribed.

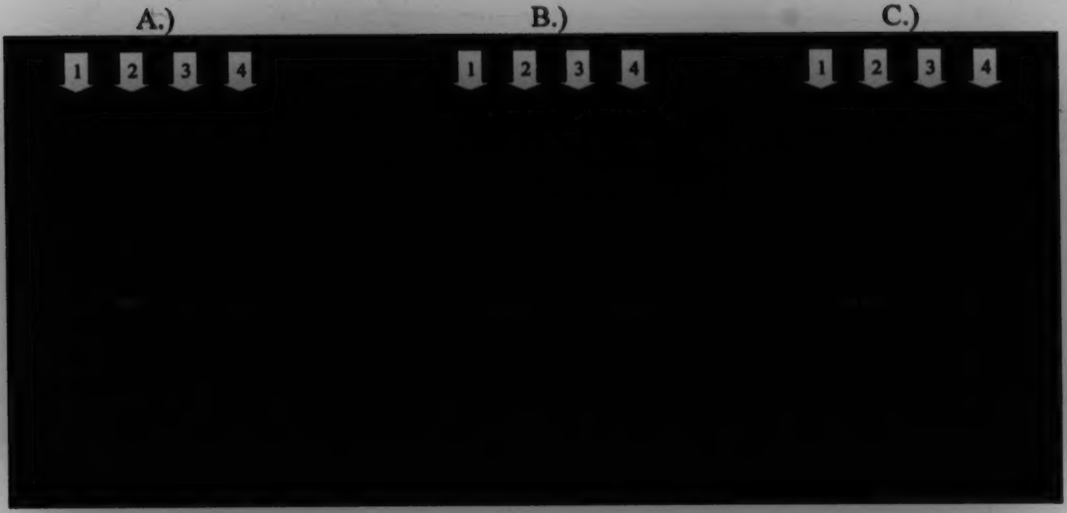


Figure 5.8. Total cellular RNA isolated from *C. maltaromaticum* UAL26 and *C. maltaromaticum* JG126 grown at 15°C and 25°C, and separated on an agarose gel to be used for Northern transfer. The gel includes three sets (A., B., C.) of the four RNA samples from each of the four cultures. Lane 1: RNA from *C. maltaromaticum* UAL26 grown at 25°C; lane 2: RNA from *C. maltaromaticum* JG126 grown at 25°C; lane 3: RNA from *C. maltaromaticum* UAL26 grown at 15°C; lane 4: RNA from *C. maltaromaticum* JG126 grown at 15°C.

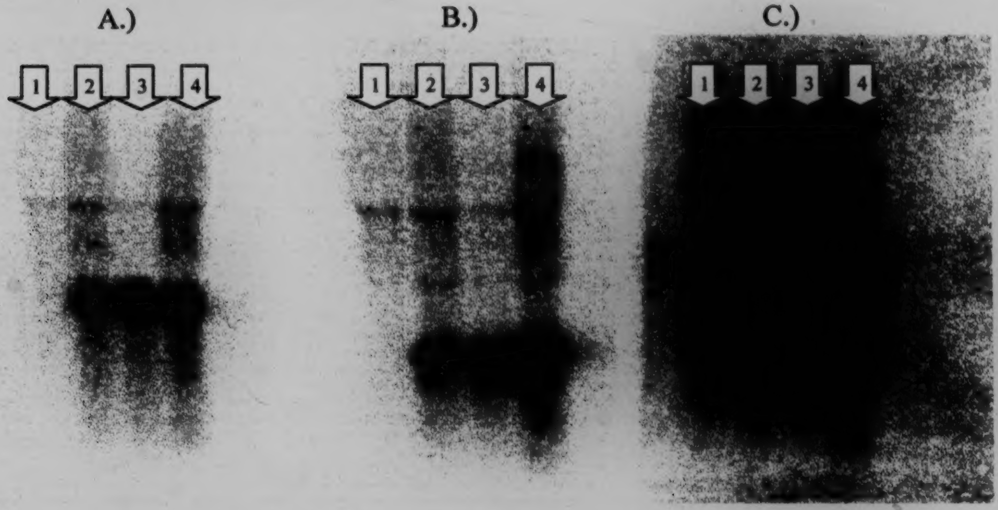


Figure 5.9. Hybridization of ^{32}P ATP-labelled DNA probes to RNA samples from *C. maltaromaticum* UAL26 and JG126 grown at 15 and 25°C. A.) piscicolin 126 probe; B.) carnobacteriocin BM1 probe; C.) 16S rRNA control probe. Lane 1: *C. maltaromaticum* UAL26 grown at 25°C; lane 2: RNA from *C. maltaromaticum* JG126 grown at 25°C; lane 3: RNA from *C. maltaromaticum* UAL26 grown at 15°C; lane 4: RNA from *C. maltaromaticum* JG126 grown at 15°C.

5.3.5. RT-PCR of the RNA samples

To compare the expression of the genes of the piscicolin 126 operon in cultures of *C. maltaromaticum* UAL26 and JG126 grown at 15°C and 25°C, RT-PCR was performed on the RNA samples isolated from these cultures. The amplified PCR products from the RT-PCR of the genes in the piscicolin 126 operon in the four different RNA samples are shown in Figure 5.10. Marker bands were not included in the figure for compactness and simplicity; however, all bands were of correct size based on the expected fragment size calculated from the sequence of the piscicolin 126 operon in *C. maltaromaticum* JG126. The results showed that for all the genes in the piscicolin 126 operon the amount of RT-PCR product was comparable for *C. maltaromaticum* UAL 26 grown at 15°C and *C. maltaromaticum* JG126 grown at 15 and 25°C. The amount of RT-PCR product in *C. maltaromaticum* UAL 26 grown at 25°C is comparably less or absent, indicating that transcription of the genes in the piscicolin 126 operon is downregulated when *C. maltaromaticum* UAL26 is grown at 25°C. Transcription of the piscicolin 126 structural gene (*pisA*) was not detected by RT-PCR, suggesting that this gene is not expressed, or expressed at very low levels in cultures of *C. maltaromaticum* UAL26 grown at 25°C. RT-PCR was performed on the RNA samples isolated from *C. maltaromaticum* UAL26, *C. maltaromaticum* UAL26 supplemented with induction peptide, and *C. maltaromaticum* JG126 grown at 25°C to compare the expression of the genes in the piscicolin 126 operon and the carnobacteriocin BM1 structural gene. The amplified PCR products from the RT-PCR are shown in Figure 5.11. The results showed that for all the genes in the piscicolin 126 operon the amount of RT-PCR product was comparable for *C. maltaromaticum* UAL 26 supplemented with synthetic induction peptide and *C.*

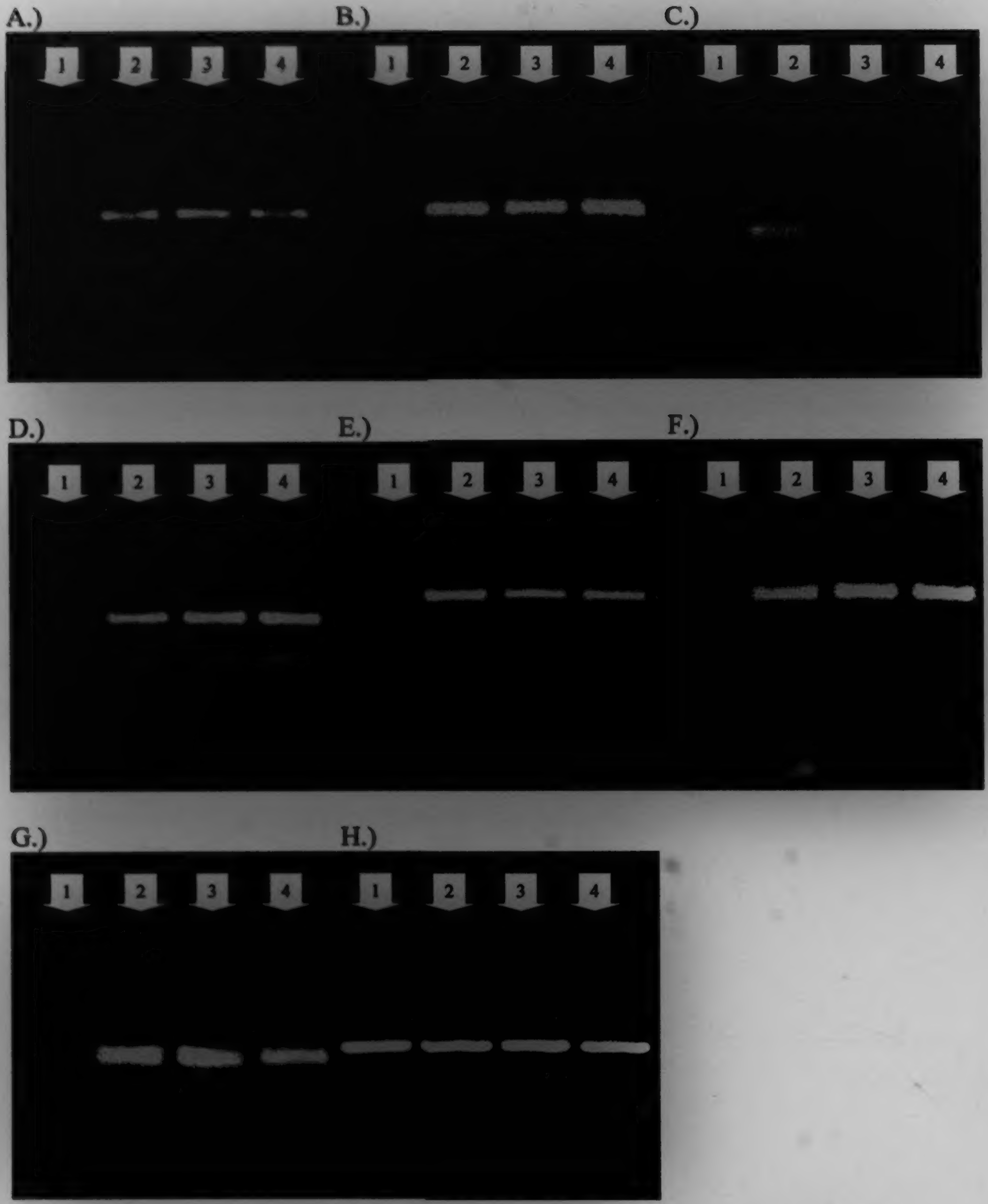


Figure 5.10. RT-PCR agarose gel electrophoresis of the genes in the piscicolin 126 operon from RNA samples isolated from *C. maltaromaticum* UAL26 and JG126. A.) *pisI*; B.) *pisA*; C.) *pisN*; D.) *pisK*; E.) *pisR*; F.) *pisT*; G.) *pisE*; H.) 16S rRNA control. Lane 1.) *C. maltaromaticum* UAL26 grown at 25°C; lane 2.) *C. maltaromaticum* JG126 grown at 25°C; lane 3.) *C. maltaromaticum* UAL26 grown at 15°C; lane 4.) *C. maltaromaticum* JG126 grown at 15°C.

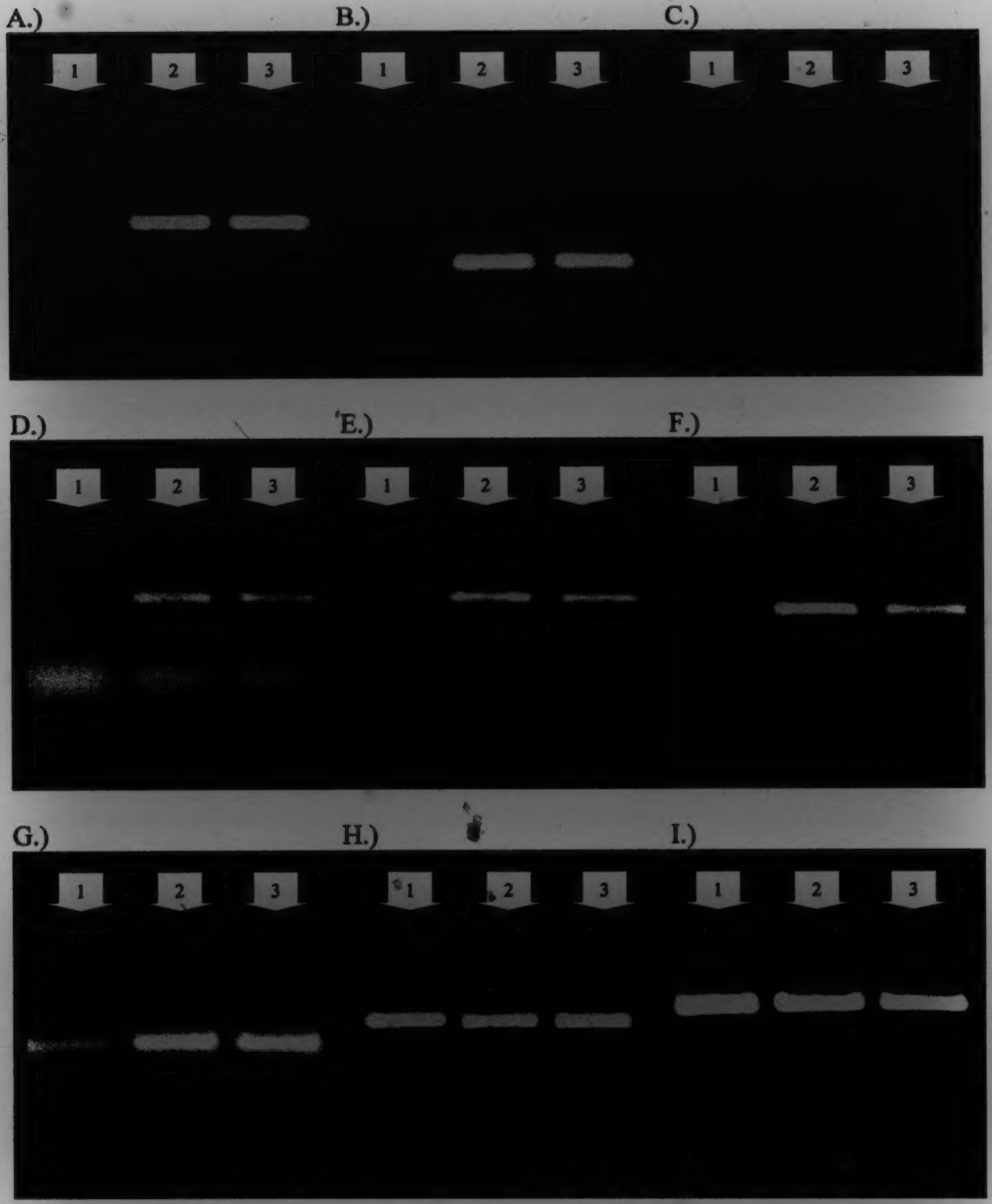


Figure 5.11. RT-PCR agarose gel electrophoresis of the genes in the piscicolin 126 and carnobacteriocin BM1 operons from RNA samples isolated from *C. maltaromaticum* UAL26 and JG126. A.) *pisI*; B.) *pisA*; C.) *pisN*; D.) *pisK*; E.) *pisR*; F.) *pisT*; G.) *pisE*; H.) *cbnBM1*; I.) 16S rRNA control. Lane 1.) *C. maltaromaticum* UAL26 grown at 25°C; lane 2.) *C. maltaromaticum* UAL26 + 5 µM induction peptide grown at 25°C; lane 3.) *C. maltaromaticum* JG126 grown at 25°C.

maltaromaticum JG126 grown at 25°C. The amount of RT-PCR product in *C. maltaromaticum* UAL26 grown at 25°C without induction peptide is less or absent. This indicates that addition of induction peptide induces transcription of the genes in the piscicolin 126 operon in cultures of *C. maltaromaticum* UAL26 grown at 25°C to amounts comparable to that observed in *C. maltaromaticum* JG126. Transcription of the carnobacteriocin BM1 structural gene (*cbnBM1*) was detected in the sample from *C. maltaromaticum* UAL26. This may indicate that carnobacteriocin BM1 is transcribed in *C. maltaromaticum* UAL26 cultures grown at 25°C, but that transcription may not occur at the same time as piscicolin 126.

5.3.6. Construction of the plasmid pLG26PISNC and transformation of *C. maltaromaticum* UAL26

An attempt was made to create a strain of *C. maltaromaticum* UAL26 that carried a plasmid containing the gene for the induction peptide of piscicolin 126. The transformation of *C. maltaromaticum* UAL26 with pLG26PISNC resulted in the growth of 24 colonies. None of the colonies had an increased amount of bacteriocin activity as compared to untransformed *C. maltaromaticum* UAL26 when assayed by spot-on-lawn inhibition at 25°C using *L. monocytogenes* ATCC 43256 as the indicator organism. While all of the colonies contained the pMG36e plasmid, none had the insert containing the functional *pisN* gene.

5.4. Discussion

The discovery that cultures of *C. maltaromaticum* UAL26 and *C. maltaromaticum* JG126 would rapidly restore bacteriocin activity to maximum titre allowed isolation of sufficient quantities of RNA for analysis. In previous experiments, it was observed that production of bacteriocin activity began in mid-log phase (Chapter 2, this thesis); however, a smaller inoculum (0.1%) was used than that used in the RNA analysis (1%). This difference may have led to faster restoration of maximum bacteriocin titre, possibly due to a higher concentration of induction peptide carried over by subculturing using a larger inoculum volume. The development of this method allowed isolation of mRNA that revealed differences in bacteriocin gene expression between two strains of *C. maltaromaticum* when grown at two temperatures. The results of the Northern analysis showed the absence of transcription of the structural genes for bacteriocin production in *C. maltaromaticum* UAL26 grown at 25°C, while the piscicolin 126 structural gene in *C. maltaromaticum* UAL26 grown at 15°C was transcribed at levels comparable to that observed in *C. maltaromaticum* JG126 grown at 15°C and 25°C. This demonstrates that temperature is a factor in the expression of piscicolin 126 genes in *C. maltaromaticum* UAL26. Other researchers (Saucier *et al.*, 1997) have shown that bacteriocin production can be dependent on inoculum size. Bacteriocin expression in *C. maltaromaticum* LV17 can be stopped by subculturing at an inoculum of less than 10^{-4} CFU/ml and a bacteriocin non-producing culture can be induced by the addition of cell-free supernatant from a bacteriocin producing culture. Northern analysis of an induced culture demonstrated transcription of carnobacteriocins A, BM1, and B2. This proved that transcription of bacteriocins in *C. maltaromaticum* LV17 is dependent on inoculum size (Saucier *et al.*, 1997). *Lactobacillus sakei* 5, which produces the bacteriocins sakacin P, T, and X, also has transcription-regulated control. The genes responsible for this are located on two separate operons, one sensitive to extracellular population (quorum sensing), and the other makes use of a histidine kinase sensory molecule and a response regulator molecule. Upon activation of either of these regulatory mechanisms, an increase in transcription of the regulatory operons is observed, followed by activation of transcription of the bacteriocin structural genes (Vaughan *et al.*, 2004). Both *C. maltaromaticum* LV17 and *L. sakei* 5 bear similarity to *C. maltaromaticum* UAL26 in that bacteriocin transcription, translation, and export commences once the required conditions to stimulate bacteriocin production are met.

RT-PCR results demonstrated the downregulation of transcription of all genes in the piscicolin 126 operon in *C. maltaromaticum* UAL26 when the culture was grown at 25°C. Transcription of the bacteriocin structural gene *pisA* was not detected. The remaining genes in the piscicolin 126 operon were transcribed but not fully expressed. Several genes (*pisR* and *pisE* in Figure 5.10, and *pisA*, *pisR*, *pisE*, and *cbnBM1* in Figure 5.11) had faint larger bands or smears of amplified PCR product. It is possible that the primers used to amplify these genes by PCR bind to other genomic DNA, most likely to genes with homologous sequence. The absence of piscicolin 126 bacteriocin mRNA supports the findings of the Northern analysis: the piscicolin 126 structural gene was not expressed in *C. maltaromaticum* UAL26 grown at 25°C. In the mRNA sample taken for RT-PCR analysis from an induced culture of *C. maltaromaticum* UAL26, mRNA from the carnobacteriocin BM1 gene was detected. This was contrary to the results observed in the Northern analysis, and indicates that carnobacteriocin BM1 may be transcribed by *C. maltaromaticum* UAL26 grown at 25°C.

Induction of bacteriocin operons is well documented, and regulation of class II bacteriocin production has been reviewed (Quadri, 2003). Methods of control by regulatory proteins in bacteriocin operons have been demonstrated, including the induction peptide (Kleerebezem *et al.*, 1997; Diep *et al.*, 2001; Kleerebezem *et al.*, 2001), the histidine kinase (Diep *et al.*, 2001), and the response regulator (Diep *et al.*, 2003). Genetic engineering of bacteriocin induction systems has allowed the development of promoter (Mathiesen *et al.*, 2004) and induction peptide (Hickey *et al.*, 2003) regulated gene expression, which may prove useful as novel bacteriocin expression systems. Bacteriocin induction in *C. maltaromaticum* LV17 is quite different from that observed in *C. maltaromaticum* UAL26 and JG126. Carnobacteriocin B2 can act as an autoinducer of its own expression although the operon also contains a gene encoding an induction peptide (Kleerebezem *et al.*, 2001). The ability to dilute the induction peptide by serial dilution of a subculture inoculum in *C. maltaromaticum* UAL26 or JG126 illustrates that the induction mechanism in *C. maltaromaticum* UAL26 and JG126 is similar to that found in *C. maltaromaticum* LV17 (Saucier *et al.*, 1995; Saucier *et al.*, 1997), and its derivative strains (Franz *et al.*, 2000).

The induction peptide in the piscicolin 126 operon plays an important role in the regulation of bacteriocin production in *C. maltaromaticum* UAL26. Supplementing broth media with spent supernatant from a culture that produces piscicolin 126 (either *C. maltaromaticum* UAL26 grown at 15°C, or *C. maltaromaticum* JG126 grown at 25°C) induces bacteriocin production within growth of the first subculture in *C. maltaromaticum* UAL26 grown at 25°C. Although bacteriocin production can be eliminated in *C. maltaromaticum* JG126 when it is grown at 35°C, the induction peptide is not able to induce bacteriocin production in a bacteriocin-negative culture under these conditions. The piscicolin 126 induction peptide was unable to induce bacteriocin production in *C. maltaromaticum* LV17. This indicates that there is no cross-induction between cultures that produce piscicolin 126 and *C. maltaromaticum* LV17. This also indicates that carnobacteriocin BM1 production in *C. maltaromaticum* LV17 is not induced by the piscicolin 126 induction peptide. The slower growth of *C. maltaromaticum* UAL26 when supplemented with supernatant from *C. maltaromaticum* LV17 may be attributed to the presence of bacteriocins produced by *C. maltaromaticum* LV17 to which *C. maltaromaticum* UAL26 has no cross-immunity.

C. maltaromaticum LV17 does not inhibit the growth of *L. monocytogenes* ATCC 43256 (results not shown). This indicates that none of the bacteriocins produced by *C. maltaromaticum* LV17 are active against this indicator organism, which is highly sensitive to piscicolin 126. As all three *Carnobacterium* strains carry the gene for carnobacteriocin BM1, it can be concluded that carnobacteriocin BM1 does not inhibit *L. monocytogenes* ATCC 43256, and any bacteriocin activity against this indicator organism is likely due to the presence of piscicolin 126. All genes in the piscicolin 126 operon in *C. maltaromaticum* UAL26 grown at 25°C with addition of synthetic induction peptide are transcribed at levels comparable to those observed in *C. maltaromaticum* JG126. This indicates that the reduced bacteriocin titre produced by *C. maltaromaticum* UAL26 supplemented with induction peptide and grown at 25°C may be due to some limitation of the export of active bacteriocin at this temperature.

Attempts to create a strain of *C. maltaromaticum* UAL26 with a plasmid that contained the gene for the piscicolin 126 induction peptide were unsuccessful. This prevented collection of *in vivo* evidence of induction of piscicolin 126 production in *C. maltaromaticum* UAL26 grown at 25°C. Attempts to transform *C. maltaromaticum* UAL26 with a plasmid containing the piscicolin 126 induction gene did not result in the isolation of viable transformants. This prevents conclusions as to the exact cause of the difference between bacteriocin expression in *C. maltaromaticum* UAL26 and *C. maltaromaticum* JG126. One approach to elucidating the difference would be to sequence the complete piscicolin 126 operon of *C. maltaromaticum* UAL26 and compare it to the sequence of the operon of *C. maltaromaticum* JG126.

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6. SEQUENCING OF THE PISCICOLIN 126 OPERON IN *Carnobacterium maltaromaticum* UAL26

6.1. Introduction

C. maltaromaticum UAL26 and JG126, two strains identified as the same species, but isolated from different sources [vacuum packaged ground beef in Canada (Burns, 1987), and spoiled ham in Australia (Jack *et al.*, 1996), respectively], contain the operons necessary to produce piscicolin 126 and carnobacteriocin BM1, yet show remarkable difference in the growth conditions necessary for bacteriocin production. Sequencing of the piscicolin 126 operon in *C. maltaromaticum* UAL26 showed that the bacteriocin structural gene, promoter region, and induction gene were of identical DNA sequence to that found in *C. maltaromaticum* JG126 (Chapter 3, this thesis), and it is known that other regions of the operon are of similar size. However, there still exists the possibility that differences reside in unsequenced regions. The remaining genes in *C. maltaromaticum* UAL26 for which no sequence data is available include the immunity, the histidine kinase, response regulator, dedicated transporter, and transport accessory genes. Differences in these genes may be involved in the effect of temperature on bacteriocin production in *C. maltaromaticum* UAL26.

Differences in the piscicolin 126 operon that cause alteration of the characteristics of bacteriocin production between *C. maltaromaticum* UAL26 and JG126 could allow genetic manipulation that may result in bacteriocin production in *C. maltaromaticum* UAL26 at 25°C. The primary objective of this research was to obtain the complete sequence of the piscicolin 126 operon in *C. maltaromaticum* UAL26 and compare it with the sequence of the piscicolin 126 operon in *C. maltaromaticum* JG126.

6.2. Materials and methods

6.2.1. Bacterial strains and growth media

C. maltaromaticum UAL26 was acquired from the University of Alberta Lactic Acid Bacteria collection. (University of Alberta, Edmonton, AB). *C. maltaromaticum* JG126 was acquired from the Australian Food Industry Science Centre Culture Collection (Werribee, Victoria, Australia).

Stock cultures of *C. maltaromaticum* UAL26 and *C. maltaromaticum* JG126 were maintained at -70°C in All Purpose Tween broth (APT; Difco; BD Diagnostics, Sparks, MD) with glycerol (20% vol./vol.; Fisher Scientific, Edmonton, AB). Cultures of *C. maltaromaticum* UAL26 and JG126 used in this study were prepared by inoculation of cells from frozen stock culture into APT broth. Cultures were then subcultured at least once more with an inoculation volume of 1% vol./vol. before use in experiments.

6.2.2. Sequencing template and primer design and preparation

Sequencing templates were prepared using 1 µl of Platinum Taq DNA polymerase High Fidelity (Invitrogen Canada Inc., Burlington, ON), 10 µl of HIFI Taq Buffer, 4 µl of 50 mM $MgSO_4$, 1 µl of each primer at 200 nM, 2 µl of 10 mM dNTP solutions (Invitrogen), and 1 µl of purified genomic DNA from *C. maltaromaticum* UAL26 isolated as described previously (Chapter 3, this thesis).

The following protocol was used on a GeneAmp PCR system 2400 (version 2.11; Perkin-Elmer Corp., Norwalk, CT): 5 min denaturation at 94°C, then 40 cycles of: 1 min denaturation at 94°C, 1 min annealing at 50°C, 3 min extension at 68°C, then a final 5 min extension at 72°C, and the samples were cooled and held at 4°C. Each template reaction mixture (20 µl) was loaded onto a 1% w./vol. agarose (BD Diagnostics) gel [dissolved in 1 x TBE buffer: 10.778 g of tris(hydroxymethyl)aminomethane (Tris; Fisher Scientific), 0.744 g of disodium ethylenediaminetetraacetic acid (EDTA; Fisher Scientific), 5.5 g of boric acid (Fisher Scientific)/L] and separated in 1 x TBE buffer by electrophoresis at 110 V for approximately 30 min using a Mini-Sub Cell GT gel box (BioRad, Hercules, CA). The gel was visualized with a Gel Doc 1000 UV transilluminator (BioRad) and photographed.

Sequencing templates were designed to be between 1.7 to 2.1 kb, with approximately 200 bp overlap between adjacent templates. The sequences of the primers used to sequence the piscicolin 126 operon are listed in Table 6.1. The primer combinations used to synthesize and sequence each template are listed in Table 6.2. Template PCR products were designed with multiple overlap of the entire piscicolin 126 operon in both directions to ensure that any differences from the piscicolin 126 operon in *C. maltaromaticum* JG126 were not the result of sequencing errors. Differences that were found were resequenced using newly amplified template to ensure that mutations were not due to errors in PCR replication of the templates used.

6.2.3. Sequencing and data processing of the piscicolin 126 operon in *C. maltaromaticum* UAL26

Sequencing reactions were performed by the Molecular Biology Services Unit (Biological Sciences, University of Alberta) using the Applied Biosystems Big Dye v3.1 dye sequencing kit (Applied Biosystems, Foster City, CA) and analysed on an AB-373 DNA Sequencer (Applied Biosystems). Sequence data was compiled using Sequencher 4.1 (Gene Codes Corporation, Ann Arbor, MI).

Table 6.1. Primers used to sequence the piscicolin 126 operon in *C. maltaromaticum* UAL26.

Primer name	Primer sequence
D1	5'-ATATGGATCCTACAGTACAACCATTTTTATTA-3'
D2E	5'-AGAGGCATGCTGAACTAATGATTGATATACT-3'
F2	5'-ATATGAATTCATGAAAACTGTTAAAGAACT-3'
FI	5'-CTAATATCCATATCTAATATTG-3'
FP	5'-AAGTTCTTTAACAGTTTTCAT-3'
NCF1	5'-AGAGATCGATAATTGGAGGGAATAAAAATGAACG-3'
PKF1	5'-AGAGGAGCTCAAGCGTACTTACTAAGAATG-3'
R2	5'-ATATTCTAGATTATCCTTTGTTCCAACC-3'
RI	5'-ATGGGTAAGTTAAAATGGTTT-3'
RN	5'-TTAGCATTTTTTAAAAAAAGAAA-3'
RP	5'-TTTTATTCCCTCCAATTTTTT-3'
U1	5'-ATATGGATCCGATTGGAGCAAAGCTATTG-3'
U2	5'-ATATGAGCTCGTTATTAGTGAATTGACAGAA-3'
U2A	5'-AGAGGAGCTCGTTATTAGTGAATTGACAGAA-3'
D2	5'-ATATCCCGGGTTAGCCGCTTCAATTGCAT-3'
D2A	5'-ATATCCCGGGTTGTTCCAACTGATCAGTG-3'
FK1	5'-TTGTAAAGTATAGTTCTATTA-3'
FK2	5'-GATAATGCAATTGAAGCGG-3'
FN	5'-ATGAACGATAAAAAATACTTG-3'
FR	5'-GAAGATCAAATCATTCAAC-3'
PKR2	5'-ACACGGATCCTTACCAAATAAGGATAATGC-3'
RK1	5'-ATGTTTAAATTTATTTAGCTTTT-3'
RK2	5'-CAATCCTAATCCTTTATGG-3'
RR	5'-TTTGAACTAACCTTTGCCG-3'
PRF1	5'-AGAGGAGCTCTGCGAAGATCAAATCATTCA-3'
PRR2	5'-ACACGGATCCCTTTAATTTGCTTTGCCTTAC-3'
TCF1	5'-AGAGATCGATATTTTGAAAGGACCGCTGTATCG-3'
TSEQF1	5'-GGCGTGTGGAAACATTA-3'
TSEQF2	5'-GCTAAAGAATGGACAGG-3
TSEQR1	5'-ATAGTCTAGAATTCCTTGA-3'
FT1	5'-GGTGTTAGTGGATCTGGA-3'
PTF1	5'-AGAGGAGCTCCATATTCAGCAACAAGATGA-3'
PTR2	5'-ACACGGATCCAATGTTGCACCAGCAACC-3'
RT1	5'-CTTCAATAAAGCACGAGCA-3'
RT2	5'-ATCCAGTCCACTGGTCGC-3'
TSEQF3	5'-AAAGCAAATCAAGAAGAAA-3'
TSEQF4	5'-GTTATATTGGGGCAACGAA-3'
TSEQF5	5'-ATCATCAAGTCGATAGAGAA-3'
TSEQR2	5'-TCAACGTTGGAGCTTTC-3'
TSEQR3	5'-TAGATGATTGATACGGCCA-3'
TSEQR4	5'-AGCAACTTGGGCTGTCTG-3'
ECF1	5'-AGAGCTGCAGAAAAGGAAGGCTAAATTATGCATAA-3'
ECR2	5'-ATATGCATGCACTTTATTCTTGATTTAGCAACACA-3'
ESEQF1	5'-GCAAGTCTTGGCTACAA-3'
ESEQF2	5'-TTGAGGGAACTCTAAAAG-3'
ESEQR1	5'-TCCTCGTACAAGCTGTTC-3'
ESEQR2	5'-TTGGTCATGTATCAATGAA-3'
FE	5'-ATGCATAATAATAACTGGCG-3'
FT2	5'-GCGACCATGGACTGGAT-3'
PEF1	5'-AGAGGAGCTCATGCATAATAATAACTGGCG-3'
PER2	5'-ACACGGATCCTTATTCTTGATTTAGCAACAC-3'
RE	5'-AGTGGTACTTGTAGTTTAT-3'

Table 6.2. Piscicolin 126 operon sequencing template and primer combinations. Seven templates were amplified from genomic DNA of *C. maltaromaticum* UAL26 by PCR. The forward and reverse strands of each template were sequenced using the sets of the primers listed.

Template	Template PCR primers	Template size (bp)	Forward sequencing primers	Reverse sequencing primers
1	U2A/D2E	~1700	D1,FI,FP,NCF1,PKF1,R2,U2,U2A	D2E,F2,RI,RN,RP,U1
2	FN/RR	~1900	FK1,FK2,FN,FR,PKF1	D2,D2A,D2E,PKR2,RK1,RK2,RR
3	PRF1/PTR2	~1700	PRF1,TCF1,TSEQF1,TSEQF2	PRR2,PTR2,RR,TSEQR1
4	PTF1/TCR2	~2100	FT1,PTF1,TSEQF2,TSEQF3, TSEQF4,TSEQF5	PTR2,RT1,RT2,TSEQR1,TSEQR2, TSEQR3,TSEQR4
5	FT1/ESEQR2	~1900	ECF1,ESEQF1,ESEQF2,FE,FT1, FT2,PEF1	ECR2,ESEQR1,ESEQR2,PER2,RE, RT1,RT2
6	TSEQF3/RT1	~900	TSEQF3,TSEQF5	TSEQR3,RT1
7	PEF1/PER2	~1000	ESEQF1,FI	ESEQR1,PER2

6.2.4. Amino acid similarity analysis, hydropathy plots, and secondary structure prediction

Amino acid similarity was done using the Zvelebil amino acid property table (Zvelebil *et al.*, 1987), assigning values for each of ten chemical properties to each amino acid to compare the degree of similarity between the piscicolin 126 operons from *C. maltaromaticum* UAL26 with those from *C. maltaromaticum* JG126.

Hydropathy plots (Kyte and Doolittle, 1982) were generated using the ProtScale engine (Gasteiger *et al.*, 2003; ExPASy, 2004) with a window size of 5, relative window edge weight of 100%, linear weight variation model, and a non-normalized scale.

Secondary structures of the proteins in the piscicolin 126 operon were predicted using the nnPredict engine (McLelland and Rumelhart, 1988; Kneller *et al.*, 1990; NnPredict, 2004) without tertiary structure class.

6.2.5. Three-dimensional modelling of the piscicolin 126 structural peptide

The three-dimensional structure of the piscicolin 126 peptide was modelled and characterized by our collaborators, Dr. Kamaljit Kaur (Department of Pharmacy, University of Alberta) and Dr. John C. Vederas (Department of Chemistry, University of Alberta) using the following protocol.

The sequence corresponding to piscicolin 126 was analysed using the SWISSPROT (Boeckmann *et al.*, 2003) and Protein Data Bank (PDB) (Berman *et al.*, 2002) databases using both FASTA (Pearson, 1990) and BLAST (Altschul *et al.*, 1990). Piscicolin 126 showed 77% sequence identity to sakacin P (PDB code 1OG7), for which the 3-dimensional nuclear magnetic resonance (NMR) structure in trifluoroethanol (TFE) has been determined (Uteng *et al.*, 2003). Multiple amino acid sequence alignment and initial homology based structure was done using the "magic fit" module of Swiss-PDBViewer 3.7 (Guex and Peitsch, 1997). This homology-based structure was chosen as the starting structure for subsequent molecular dynamics (MD) simulations. The structure consisted of 43 amino acids of the piscicolin 126 sequence, without the last glycine residue. MD simulations of solvated bacteriocin in TFE were performed using a parallel version of the GROMACS 3.1 molecular dynamics simulation package (GROMOS96 forcefield) (Spoel *et al.*, 2002) on a dual Pentium III personal computer processor machine running LAM-MPI version 6.5.4 (Gropp *et al.*, 1999). TFE solvent was parameterized (Fioroni *et al.*, 2000), which attributed the following charges to the TFE atoms: F = –0.17 e, C = 0.452 e, CH2 = 0.273 e, OA = –0.625 e, and HO = 0.410 e (where OA and HO are the hydroxylic oxygen and hydrogen atoms, respectively, for the GROMOS forcefield).

The system was simulated in an NPT ensemble at 298 K using periodic boundary conditions. Weak coupling of the protein to a solvent bath of constant temperature was maintained using the Berendsen thermostat (Berendsen *et al.*, 1984) with a coupling constant of $\tau_T = 0.1$ ps. The pressure was controlled using the Berendsen algorithm at 1 bar with a coupling constant $\tau_P = 4$ ps, using a compressibility of liquid TFE of 1.22×10^{-4} kJ^{-1} mol nm^3 at 298 K (Fioroni *et al.*, 2000). The electrostatic and van der Waal's interactions were truncated at a cut-off distance of 1.2 nm. The integration time step was 2 fs, and the coordinates and velocities were saved every 2 ps. The linear constraint solver algorithm was used to restrain all bond lengths (Hess *et al.*, 1997).

Piscicolin 126 was considered to have positively and negatively charged N-terminal NH_3^+ and C-terminal COO^- groups. Lysine residues were considered to be charged. Piscicolin 126 had a total final charge of +4 e. The simulation system was generated by peptide insertion into a cubic box with an edge length of ~6 nm, followed by solvation of the protein using 1512 TFE molecules. The system was initially subjected to 1000 steps of steepest descent energy minimization. The protein coordinates of the resulting system were then restrained while allowing solvent molecules to relax their positions and optimize interactions with the protein during a 200 ps simulation. After initial equilibration, a 5 ns MD simulation was run. Snapshots of the peptide structure extracted from the last 1 ns of the MD simulation were averaged and minimized further using a conjugate gradient algorithm. A measure of the overall peptide stability was obtained by plotting the root-mean-square deviation (RMSD) of the peptide structure fitted onto its initial structure as a function of time (Figure 6.5). The final RMSD for all

Cα atoms of the bacteriocin averaged over the last 1 ns of the simulation was 0.35 ns. Simulations were analyzed using GROMACS routines.

6.2.6. Construction of the plasmids pLG26PISTC and pLG26PISEC, and transformation of *C. maltaromaticum* UAL26

DNA primers (Applied Biosystems 394 DNA/RNA Synthesizer; DNA Core Facility, University of Alberta) were designed based upon the sequence of the piscicolin 126 ABC-transporter gene (pLG26PISTC) and the transport accessory gene (pLG26PISEC) from *C. maltaromaticum* JG126 (Jack *et al.*, 1996), with a 10 bp sequence at the beginning (5'-end) to allow the appropriate restriction endonucleases to cut the PCR amplified DNA fragment. The primers were used in a PCR reaction (Sambrook and Russell, 2001) to amplify the piscicolin 126 transport and accessory genes. The primers used to amplify the *pisT* gene (PISTCF1 and PISTCR2) were designed to contain the *Cla*I and *Pst*I restriction sites at either end of the amplified region, while the primers used to amplify the *pisE* gene (PISECF1 and PISECR2) contained the *Pst*I and *Sph*I restriction sites. The sequence of the primers was as follows: primer PISTCF1 5'-AGAGATCGATATTTTGAAAGGACCGCTGTATCG-3', primer PISTCR2 5'-ATATCTGCAGTTAAATATCCCATAACTGTTGATAAA-3', primer PISECF1 5'-AGAGCTGCAGAAAAGGAAGGCTAAATTATGCATAA-3' and primer PISECR2 5'-ATATGCATGCACTTTATTCTTGATTTAGCAACACA-3'.

For all reactions, DNA was amplified in 100 µl PCR reactions using 0.5 µl of Platinum Taq DNA polymerase High Fidelity (Invitrogen), 10 µl of 10 x HIFI Platinum Taq Buffer, 4 µl of 50 mM $MgSO_4$, 1 µl of each primer at 200 nM (pairings of the primers listed above), 2 µl of 10 mM dNTP solutions (Invitrogen), and 1 µl of DNA template isolated from *C. maltaromaticum* JG126 prepared using the following protocol: cell culture (1 ml) was centrifuged (model 5417C; Eppendorf, Hamburg, Germany) at 20,000 x *g* for 5 min, the supernatant was discarded, and the cell pellet was resuspended in 0.5 ml sterile Milli-Q water. The cells were centrifuged again, resuspended in 100 µl sterile Milli-Q water, and heated at 100°C for 5 min.

The following PCR protocol was used on a GeneAmp PCR system 2400 thermal cycler (version 2.11; Perkin Elmer Corp.): 2 min denaturation at 94°C, 40 cycles of: 1 min denaturation at 94°C, 1 min annealing at 60°C, 2 min extension at 68°C, then a final 5 min extension at 72°C, and the samples were cooled and held at 4°C. Each reaction mixture (10 µl) was loaded onto a 1% w./vol. agarose (BD Diagnostics) gel, separated by electrophoresis, and visualized as described in section 6.2.2.

PCR amplified fragments were cleaned using QiaQuick PCR purification columns (Qiagen Inc., Hilden, Germany), eluted with a final volume of 50 µl, and then digested with the appropriate restriction endonucleases (*pisT*: *Cla*I and *Pst*I in 10 x React 1, each 10% vol./vol., *pisE*: *Pst*I and *Sph*I in 10 x React 1, each 10% vol./vol.). Plasmid vector pMG36e (previously isolated; Chapter 4, this thesis) was cut using the same protocol, without the QiaQuick purification step. The mixtures were incubated at 37°C for 2 h and purified by agarose gel electrophoresis using a gel extraction kit (Invitrogen) according to the manufacturer's protocol. Ligation was achieved by adding 10 µl of 5 x ligation buffer and 5 µl of T4 DNA ligase enzyme to each mixture. The solutions were mixed by vortex and incubated at 25°C for 2 h. The ligation mixture (10 µl) was transferred into an electroporation cuvette (BioRad) and left on ice for 2 min, then electroporated using a Gene Pulser Electroporator (BioRad) at 2.5 kV and 200 Ω resistance. APT supplemented with 0.5 M sucrose and 20 mM $MgCl_2$ (1 ml) was added to the cuvette and the mixture was transferred to a microcentrifuge tube. The culture was incubated at 25°C for 4 h, plated in 100 μl volumes onto APT agar plates containing erythromycin (5 μg/ml; Fisher Scientific), and incubated at 25°C. Colonies which grew within 6 d were picked from the agar plates, grown in APT broth with erythromycin (5 μg/ml) at 25°C for 24 h. Cell cultures (1 ml) were centrifuged at 20,000 x *g* for 2 min, the supernatant was discarded, and the cells were resuspended in 250 μl ice-cold STE (10 mM TrisCl, 0.1 M NaCl, and 1 mM EDTA, pH 8) buffer. The centrifugation was repeated and the cells were lysed by alkaline lysis (Sambrook and Russell, 2001), and extracted by vortexing for 10 s with 500 μl of buffer-saturated phenol. This mixture was separated by centrifuging at 10,000 x *g* for 1 min, and the aqueous layer transferred to a new microcentrifuge tube. An equal volume of chloroform:isoamyl alcohol (24:1) was added to the tube, and separated by centrifuging at 10,000 x *g* for 1 min. The aqueous layer was transferred to a new tube. Purified pLG26PISTC DNA was cut with the appropriate restriction enzymes for 1 h as described above, loaded onto a 1% w./vol. agarose gel, separated by electrophoresis, and visualized as described in section 6.2.2.

6.3. Results

6.3.1. Sequencing reactions and data processing of the piscicolin 126 operon in *C. maltaromaticum* UAL26

A total of 6767 bp of sequence from the piscicolin 126 operon was obtained from analysis of data from 74 sequencing reactions, using 7 overlapping templates and 52 primers. When the sequence was compared with that of *C. maltaromaticum* JG126, 34 single bp differences were found (Table 6.3). These differences included 8 single bp

Table 6.3. Summary of differences between the sequence of the piscicolin 126 operon in *C. maltaromaticum* UAL26 and the sequence of the piscicolin 126 operon in *C. maltaromaticum* JG126 (Jack *et al.*, 1996; Gibbs *et al.*, 2000).

Type	#	Base pair location	JG126:UAL26 base difference	Codon Position	JG126:UAL26 a.a. difference	Gene	Comment
Non-ORF	1	1089	T : C				
	2	1139	G : A				
	3	1201	A : C				
	4	1202	A : T				
	5	1211	C : T				
	6	1250	G : A				
	7	1591	T : A				
	8	7289	T : G				Non-ORF due to frameshift
Silent	1	2356	T : A	3		*pisN*	
	2	3418	G : T	3		*pisK*	
	3	5110	A : T	3		*pisT*	
	4	5356	G : C	3		*pisT*	
	5	5386	C : T	3		*pisT*	
	6	5941	C : T	3		*pisT*	
	7	5947	G : A	3		*pisT*	
	8	6160	T : C	3		*pisT*	
	9	6199	C : T	3		*pisT*	
	10	6233	T : C	1		*pisT*	
	11	6406	C : T	3		*pisT*	
	12	7155	G : A	3		*pisE*	
Non-silent	1	1418	C : T	1	G : S	*pisI*	
	2	2953	G : A	3	M : I	*pisK*	
	3	4801	T : A	3	N : K	*pisT*	
	4	4957	A : T	3	L : F	*pisT*	
	5	4982	T : C	1	F : L	*pisT*	
	6	5084	G : A	1	A : T	*pisT*	Same codon as 5086
	7	5086	T : A	3	A : T	*pisT*	Same codon as 5084
	8	5171	A : T	1	I : L	*pisT*	
	9	5900	C : T	1	P : S	*pisT*	
	10	5905	C : A	3	D : E	*pisT*	
	11	5916	C : T	2	T : I	*pisT*	
	12	5933	C : A	1	Q : K	*pisT*	
	13	7196	C : A	1	P : T	*pisE*	In truncated UAL26 gene after 8th frameshift residue (3rd T).
Frameshift	1	7175	C : -			*pisE*	Base pair deletion at residue 171. First 174 residues are identical. Remaining residues: TKKKITITFKI

differences which did not reside in open reading-frames (non-ORF), 12 single bp changes that would not alter the translated amino acid sequence (silent difference), 13 single bp differences which would alter the translated amino acid sequence (non-silent), and one frameshift caused by the absence of a single bp in *C. maltaromaticum* UAL26. A comparison of the differences between the piscicolin 126 operon *C. maltaromaticum* UAL26 and *C. maltaromaticum* JG126 is included in the Appendix. The differences are shown in Figure 6.1.

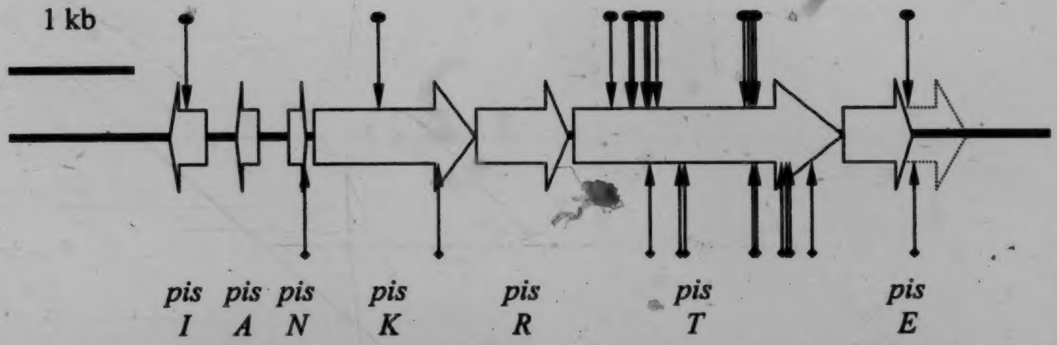


Figure 6.1. Differences in the piscicolin 126 operon in *C. maltaromaticum* UAL26 compared with the sequence of the piscicolin 126 operon in *C. maltaromaticum* JG126. ***pisI***: immunity gene; ***pisA***: structural gene; ***pisN***: induction gene; ***pisK***: histidine kinase gene; ***pisR***: response regulator gene; ***pisT***: dedicated transport gene; ***pisE***: transport accessory gene. ⟶: silent bp difference; •⟶: nonsilent single bp difference; ⇨: ***pisE*** gene in *C. maltaromaticum* JG126. Differences residing in non-ORF regions are not shown.

The frameshift in the *pisE* transport accessory gene in the piscicolin 126 operon in *C. maltaromaticum* UAL26 caused a premature truncation of the gene. Absence of a single cytosine bp at residue 7175 changed the reading frame of the remainder of the gene, resulting in a different amino acid sequence. The new reading frame coded the next 4 amino acids as identical to those in *C. maltaromaticum* JG126, and the following 3 amino acids were different. An additional single bp difference at residue 7196 changed the amino acid for this codon in *C. maltaromaticum* UAL26 to threonine, as found in *C. maltaromaticum* JG126. The next three amino acids were different, and the twelfth codon after the frameshift encoded a translation stop signal, which resulted in truncation of the protein (Figure 6.2).

6.3.2. Amino acid similarity analysis, hydropathy plots, and secondary structure prediction

Changes in the properties of the amino acids in the piscicolin 126 operon in *C. maltaromaticum* UAL26 compared with those found in *C. maltaromaticum* JG126 are summarized in Table 6.4. Most of the differences showed a slight increase in molecular size, or a change from a non-polar residue to a polar one. Only one difference resulted in alteration of charge, with a glutamine residue in *C. maltaromaticum* JG126, and a lysine residue in *C. maltaromaticum* UAL26.

A comparison of the amino acid sequence of the piscicolin 126 operon in *C. maltaromaticum* UAL26 and in *C. maltaromaticum* JG126 showed that the hydropathy of most of the areas containing different residues did not appear to change a predicted hydrophobic region (Score > 0) into a hydrophilic region (Score < 0), or vice-versa. The differences found in hydrophobicity of the amino acid sequences of the dedicated transport (PisT) and transport accessory (PisE) proteins showed the greatest differences between *C. maltaromaticum* UAL26 and JG126 (Figure 6.3). Hydropathy plots for the other proteins in the piscicolin 126 operon that had differences in amino acid sequence (PisI, PisK) showed very slight differences in hydropathy (results not shown).

```
J        #1    ATG CAT AAT AAT AAC TGG CGC GAC CAC TCC AGT GTA TAT AGT CAA CAG CAT CAA AAA
                M   H   N   N   N   W   R   D   H   S   S   V   Y   S   Q   Q   H   Q   K
U        #1    ATG CAT AAT AAT AAC TGG CGC GAC CAC TCC AGT GTA TAT AGT CAA CAG CAT CAA AAA
                M   H   N   N   N   W   R   D   H   S   S   V   Y   S   Q   Q   H   Q   K
               ...........................................................................

J        #58   TTT TAT CGT TGG GTG TTA TAT CCT GTA ATT CTT TTT TTG TTT TTG GTT AGT TTA TTT
                F   Y   R   W   V   L   Y   P   V   I   L   F   L   F   L   V   S   L   F
U        #58   TTT TAT CGT TGG GTG TTA TAT CCT GTA ATT CTT TTT TTG TTT TTG GTT AGT TTA TTT
                F   Y   R   W   V   L   Y   P   V   I   L   F   L   F   L   V   S   L   F
               ...........................................................................

J        #115  TTG GCT TTT GCG AAA AAA GAA GTT GTC GTT CGG GTA CCT GCT CAA CTA ACC GCA ACT
                L   A   F   A   K   K   E   V   V   V   R   V   P   A   Q   L   T   A   T
U        #115  TTG GCT TTT GCG AAA AAA GAA GTT GTC GTT CGG GTA CCT GCT CAA CTA ACC GCA ACT
                L   A   F   A   K   K   E   V   V   V   R   V   P   A   Q   L   T   A   T
               ...........................................................................

J        #172  AAA ATA GAT AAA CTA CAA GTA CCA CTG GAA GCA AAC ATC AAA GTA AAT AAT CTT AAA
                K   I   D   K   L   Q   V   P   L   E   A   N   I   K   V   N   N   L   K
U        #172  AAA ATA GAT AAA CTA CAA GTA CCA CTG GAA GCA AAC ATC AAA GTA AAT AAT CTT AAA
                K   I   D   K   L   Q   V   P   L   E   A   N   I   K   V   N   N   L   K
               ...........................................................................

J        #229  GAA AAT AAA GAG GTC AAA AAA GGA GAA GTT TTA GTG ATG TTT GAT ACT AGT GTT TTA
                E   N   K   E   V   K   K   G   E   V   L   V   M   F   D   T   S   V   L
U        #229  GAA AAT AAA GAG GTC AAA AAA GGA GAA GTT TTA GTG ATG TTT GAT ACT AGT GTT TTA
                E   N   K   E   V   K   K   G   E   V   L   V   M   F   D   T   S   V   L
               ...........................................................................

J        #286  CAA AAT GAA AAG GAG CAA TTT GAA CAA GAA AAT ACG TCT CTT GAA GAC CAA AAG AAA
                Q   N   E   K   E   Q   F   E   Q   E   N   T   S   L   E   D   Q   K   K
U        #286  CAA AAT GAA AAG GAG CAA TTT GAA CAA GAA AAT ACG TCT CTT GAA GAC CAA AAG AAA
                Q   N   E   K   E   Q   F   E   Q   E   N   T   S   L   E   D   Q   K   K
               ...........................................................................

J        #343  GCT GCG CAA CTA TTT ATT GAA AGT ATA GCT AAA GAA CAG AAC TTA TTT GAA GAC GAG
                A   A   Q   L   F   I   E   S   I   A   K   E   Q   N   L   F   E   D   E
U        #343  GCT GCG CAA CTA TTT ATT GAA AGT ATA GCT AAA GAA CAG AAC TTA TTT GAA GAC GAG
                A   A   Q   L   F   I   E   S   I   A   K   E   Q   N   L   F   E   D   E
               ...........................................................................

J        #400  GAT GAA TTT GGA TAC AGT AAT CAG CTA AAG AGT TTA TTA CTC GAA AAG GAA ACA CAA
                D   E   F   G   Y   S   N   Q   L   K   S   L   L   L   E   K   E   T   Q
U        #400  GAT GAA TTT GGA TAC AGT AAT CAG CTA AAG AGT TTA TTA CTC GAA AAG GAA ACA CAA
                D   E   F   G   Y   S   N   Q   L   K   S   L   L   L   E   K   E   T   Q
               ...........................................................................

J        #457  AAT ACA GAG CAA GTC TTG GCT ACA ACC AAG CAA AAG ATA GCC GTA CTA ATT GAA ACC
                N   T   E   Q   V   L   A   T   T   K   Q   K   I   A   V   L   I   E   T
U        #457  AAT ACA GAG CAA GTC TTG GCT ACA ACC AAG CAA AAG ATA GCC GTA CTA ATT GAA ACA
                N   T   E   Q   V   L   A   T   T   K   Q   K   I   A   V   L   I   E   T
               ...........................................................................
                                                                                         *
J        #514  AAA AAA AAA AAT AAC TAT ACC CTT CAA AAT ATA ACT GAA CAG CTT GTA CGA GGA ACA
                K   K   K   N   N   Y   T   L   Q   N   I   T   E   Q   L   V   R   G   T
U        #514  AAA AAA AAA ATA ACT ATA ACC TTC AAA ATA TAA
                K   K   K   I   T   I   T   F   K   I   .
               ...........................................................................

                            **  ** ***     * * *    ** **
J        #571  TTA AAA TCT CCT TCA ACT GGA ATA GTT CAC TTG AAT GAG GAA GTA ACA GGC CAA ATA
                L   K   S   P   S   T   G   I   V   H   L   N   E   E   V   T   G   Q   I
               ...........................................................................

J        #628  GAT GTA TCA AAA GGA ACG GTA CTT GCA GAA ATC TAT CCA AAG CAA AAA GAA TCA CAG
                D   V   S   K   G   T   V   L   A   E   I   Y   P   K   Q   K   E   S   Q
               ...........................................................................

J        #685  CTA ATG TTT ACT GCG CTA TTA CCT GCT GAT GAA GTA ACA CGA ATA AAA GTA GGT ATG
                L   M   F   T   A   L   L   P   A   D   E   V   T   R   I   K   V   G   M
               ...........................................................................

J        #742  GAC GTT CAT TTT AAA TTA GAT AAA AAA GGT GTT GCA TCC CAA ACA ATT GAG GGA ACT
                D   V   H   F   K   L   D   K   K   G   V   A   S   Q   T   I   E   G   T
               ...........................................................................

J        #799  CTA AAA GAG ATT TCA GAG ACT AGT ATG ACA ACT GAA CAA GGA ACC TTC TAC ACA ATA
                L   K   E   I   S   E   T   S   M   T   T   E   Q   G   T   F   Y   T   I
               ...........................................................................

J        #856  AAA GGT ACG TTA CAA TCT TCA AAA TAT TTT AAT AAT AGA TAT GGT ATG ACA GGG GAA
                K   G   T   L   Q   S   S   K   Y   F   N   N   R   Y   G   M   T   G   E
               ...........................................................................

J        #913  GTT TCG CTC ATT ATT GGA AAA AAA AGC TAT TGG CAA CAA ATC AAA GAT GTG TTG CTA
                V   S   L   I   I   G   K   K   S   Y   W   Q   Q   I   K   D   V   L   L
               ...........................................................................

J        #970  AAT CAA GAA TAA
                N   Q   E   .
               ...............
```

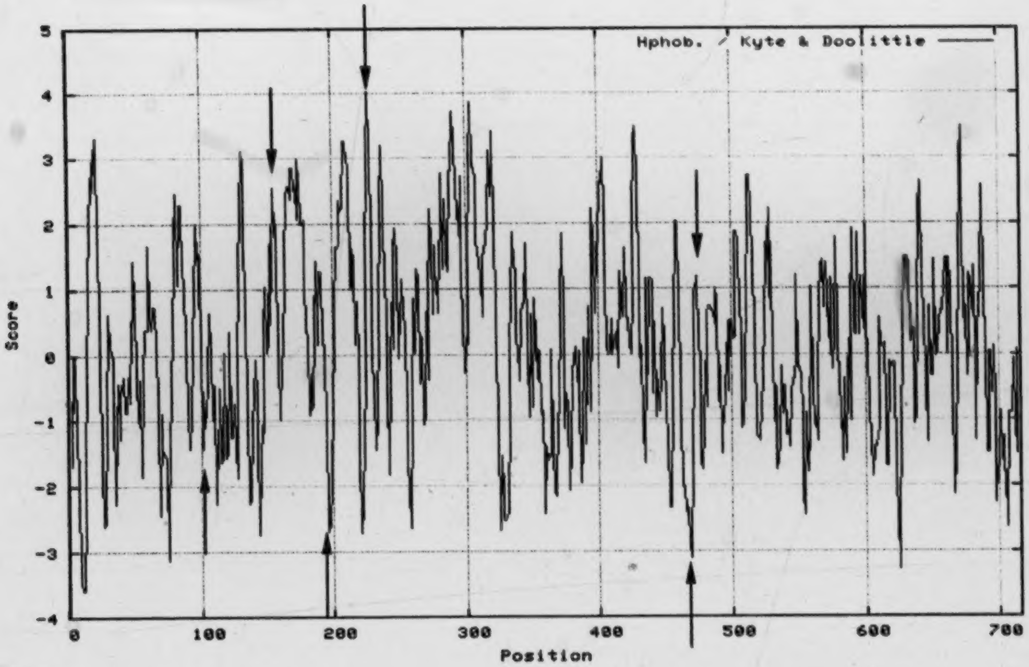
Figure 6.2. Nucleotide sequence and corresponding protein sequence of the *pisE* genes in *C. maltaromaticum* UAL26 (U) and *C. maltaromaticum* JG126 (J).

Table 6.4. Amino acid similarity properties for the differences in amino acid sequence found in the piscicolin 126 operon in *C. maltaromaticum* UAL26 and *C. maltaromaticum* JG126.

	Gene	a.a. position	JG126:UAL26 difference		Characteristic change
1	*pisI*	52/98	G : S	Glycine : Serine	Hydrophobic, Tiny : Polar, Tiny
2	*pisK*	180/428	M : I	Methionine : Isoleucine	Hydrophobic : Hydrophobic, Aliphatic
3	*pisT*	102/716	N : K	Asparagine : Lysine	Polar, Small : Hydrophobic, Positive, Polar
4	*pisT*	154/716	L : F	Leucine : Phenylalanine	Hydrophobic, Aliphatic : Hydrophobic, Aromatic
5	*pisT*	163/716	F : L	Phenylalanine : Leucine	Hydrophobic, Aromatic : Hydrophobic, Aliphatic
6	*pisT*	197/716	A : T	Alanine : Threonine	Hydrophobic, Tiny : Hydrophobic, Polar, Small
7	*pisT*	197/716	A : T	Alanine : Threonine	Hydrophobic, Tiny : Hydrophobic, Polar, Small
8	*pisT*	226/716	I : L	Isoleucine : Leucine	Hydrophobic, Aliphatic : Hydrophobic, Aliphatic
9	*pisT*	470/716	P : S	Proline : Serine	Small, Cyclic : Polar, Tiny
10	*pisT*	471/716	D : E	Aspartic acid : Glutamic acid	Negative, Polar, Small : Negative, Polar
11	*pisT*	475/716	T : I	Threonine : Isoleucine	Hydrophobic, Polar, Small : Hydrophobic, Aliphatic
12	*pisT*	481/716	Q : K	Glutamine : Lysine	Polar : Hydrophobic, Positive, Polar
13	*pisE*	178/181	P : T	Proline : Threonine	Small, Cyclic : Hydrophobic, Polar, Small

A computer neural network (NnPredict, 2004) was used to analyse the protein sequence of each gene in the piscicolin 126 operon and to predict the secondary structure of the proteins. Changes in predicted secondary structure were found as a result of single bp differences between the nucleotide sequence of the piscicolin 126 operons found in *C. maltaromaticum* UAL26 and *C. maltaromaticum* JG126. Differences found in secondary structure of the dedicated transport (PisT) and transport accessory (PisE) proteins are shown in Figure 6.4. Predicted α-helical and β-sheet structures are observed in regions consisting of multiple residues predicted to have similar structure (e.g. AAAAA... or BBBBB...). Those residues not predicted to be in either α-helix or β-sheet were assumed to be part of turn elements in protein folding.

A.)


Hphob. / Kyte & Doolittle
Score
Position
5
4
3
2
1
0
-1
-2
-3
-4
0
100
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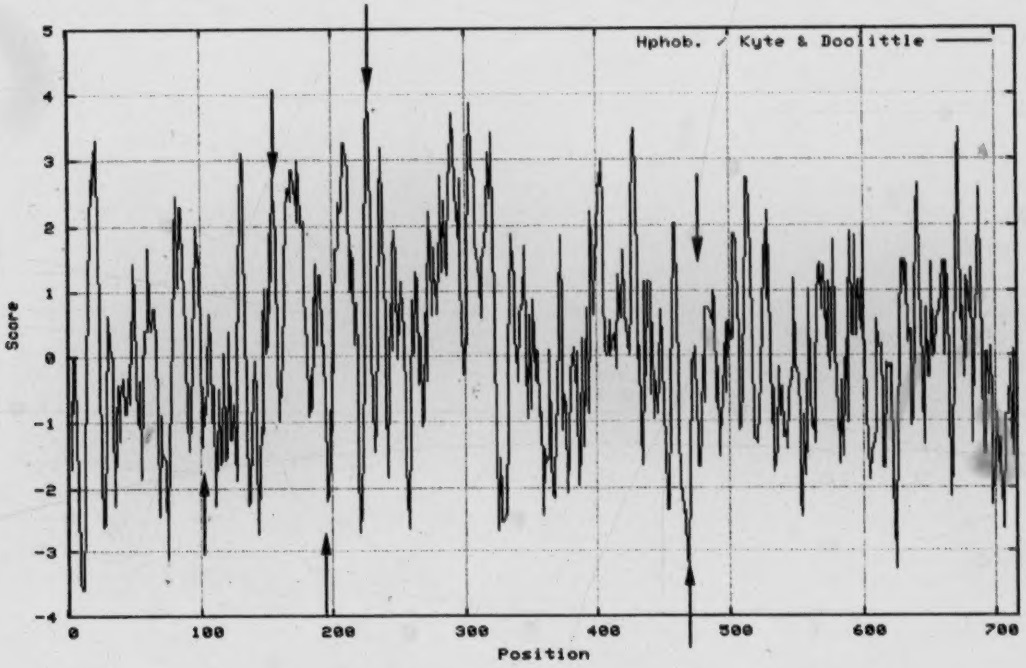
700

B.)


Hphob. / Kyte & Doolittle
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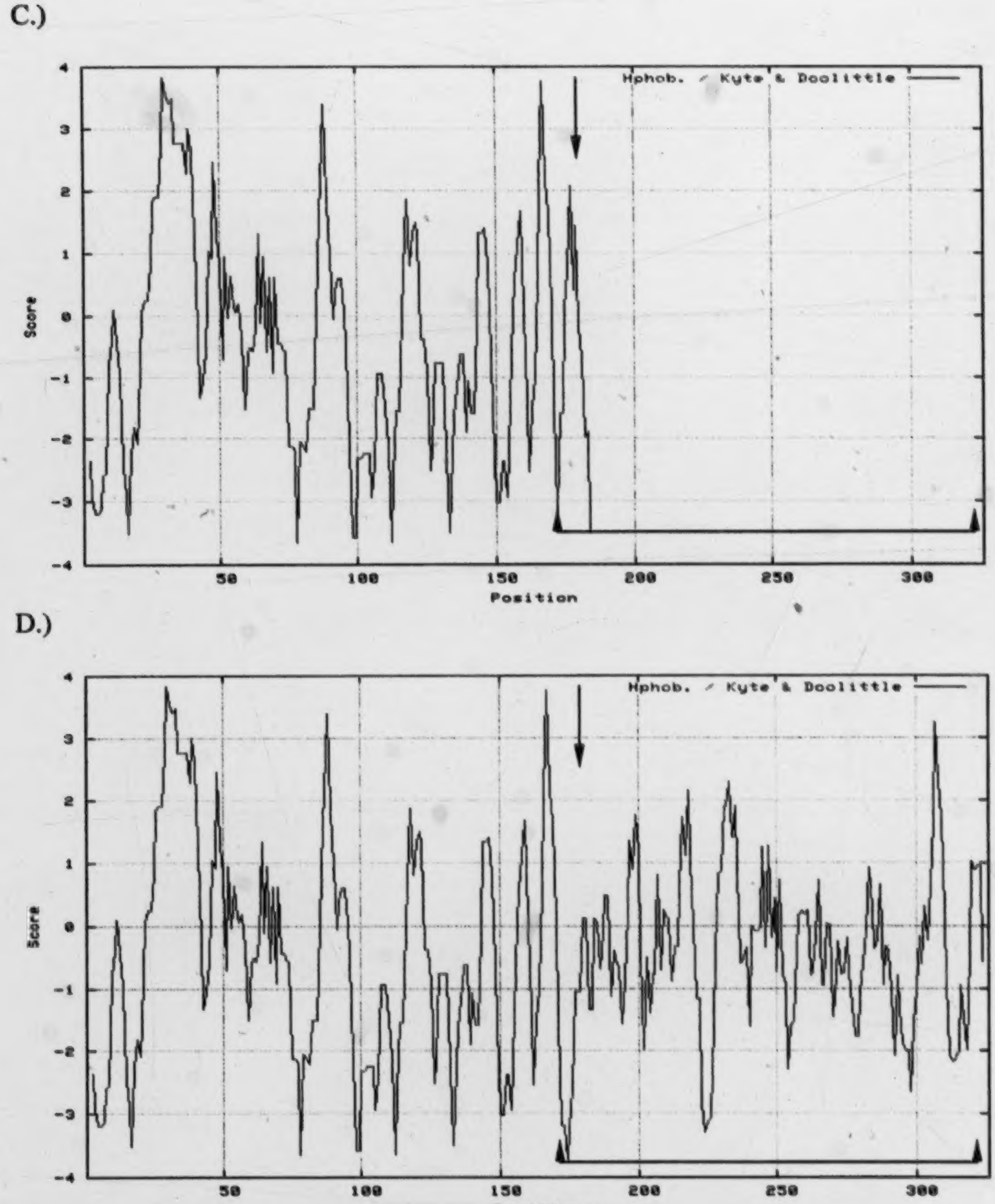

Figure 6.3. Hydropathy analysis of proteins from the piscicolin 126 operon in *C. maltaromaticum* UAL26 and JG126. A.) PisT in *C. maltaromaticum* UAL26; B.) PisT in *C. maltaromaticum* JG126; C.) PisE in *C. maltaromaticum* UAL26; D.) PisE in *C. maltaromaticum* JG126. Regions with score values above zero are hydrophobic in character, regions with score values below zero are hydrophilic in character. ——►: site of hydropathic difference.

A.)

```
MKQFTHIQQQDEKDCGVACIAMILKHYKTEIPIHKLRDFSGTDLEGTSAFGLKKTFEKLFFDCPAIQADNDVWKEKELPLPLIAHILIDN
------A----------AAAAAAAA-------AA--------------AA-AAAAAAAAA------------A--------AAABBBB--
------A----------AAAAAAAA-------AA--------------AA-AAAAAAAAA------------A--------AAABBBB--
MKQFTHIQQQDEKDCGVACIAMILKHYKTEIPIHKLRDFSGTDLEGTSAFGLKKTFEKLFFDCPAIQADNDVWKEKELPLPLIAHILIDN

SYMHYVVVYKINGETLSIDDPARGKIKKTVEEFAKEWTGILLLPTPKDSYVPSKEKVARISSFLPVIWLQKGFVFHIIVASIFITLFGIG
---BBBBBBBB-----------------AAAAAAAAAAABB--------------AAABA-----BBBA----BBBBBBBBBBBBBB---
---BBBBBBB------------------AAAAAAAAAAABB--------------AAABB-----BBBAA---BBBBBBBBBBBBBB---
SYMHYVVVYKIKGETLSIDDPARGKIKKTVEEFAKEWTGILLLPTPKDSYVPSKEKVARISSFFPVIWLQKGLVFHIIVASIFITLFGIG

SSYYFQGILDYFIPNHARSTLNIVSVGLIVVYLFRVMFEYSRNYLIVILGQRMSMVVMLQYFKHILSLPMNFFATRKSGEIISRFLDANK
--BBBB----BB--------BBBBBB-BBBBABAAAAAAA----BBBBA-----ABAAAAAAAAAA------A------ABBBAAA---A
--BBBB----BB--------BBBBBB-BBBBABAAAAAAA---ABBBBA-----ABAAAAAAAAAA------A------ABBBAAA---A
SSYYFQGILDYFIPNHTRSTLNIVSVGLIVVYLFRVMFEYSRNYLLVILGQRMSMVVMLQYFKHILSLPMNFFATRKSGEIISRFLDANK

IIDALASATLSVFLDIGMVLIVGITLAVQNGILFLITLASLPFYLVSILAFVKSYEKANQEEMVAGATLNSSIIESLKGIETIKAYSGEE
AAAAAAAAAABBBAAA-ABBBBBBBBA-A--AABBBBA----AABBABAAAAAAA----AAAAAA-------AAABA---BBBB-----A
AAAAAAAAAABBBAAA-ABBBBBBBBA-A--AABBBBA----AABBABAAAAAAA----AAAAAA-------AAABA---BBBB-----A
IIDALASATLSVFLDIGMVLIVGITLAVQNGILFLITLASLPFYLVSILAFVKSYEKANQEEMVAGATLNSSIIESLKGIETIKAYSGEE

KVYHQVDREFVQLMKKSFRTATLDNVQQGIKHAIQLISSALILWLGSYYVMEGSISLGQLITYNALLVFFTDPLQNIINLQVKMQTAQVA
AAAAAA-AAAAAAAAAA--------AAAAAAAAAAAAAAAABBB----BBBB------BBBAAAABBBB-------AAAAAA-AAAAAAA
AAAAAA-AAAAAAAAAA--------AAAAAAAAAAAAAAAABBB----BBBB------BBBAAAABBBB-------AAAAAA-AAAAAAA
KVYHQVDREFVQLMKKSFRTATLDNVQQGIKHAIQLISSALILWLGSYYVMEGSISLGQLITYNALLVFFTDPLQNIINLQVKMQTAQVA

NKRLNEIFAIEPESTNQHPDRVVTNQTFQQGITLEGVSFSYNMKAPTLSNLSCTILPHSKVALVGVSGSGKSTMAKLLVNFYPPSEGTIR
AA-A-AABB--------------------B-BBBB------------------BB------BBBBB------AAAAABB---------BB
AA-A-AABB-----------BBBB--------BBB------------------BB------BBBBB------AAAAABB---------BB
NKRLNEIFAIEPESTNQHSERVVINQTFQKGITLEGVSFSYNMKAPTLSNLSCTILPHSKVALVGVSGSGKSTMAKLLVNFYPPSEGTIR

YGRINHLDIPFHQLRDRVTYIPQESFFFSGSIIENLTFSLDTPPSFERIMEVCDAVQLGTFINQQPLRYDTILEEGGTNISGGQRQRLAI
B------------------------BBB---BBBBAB-----------AAAAA-AAA--------------BBA----B------AAAAA
B------------------------BBB---BBBBAB-----------AAAAA-AAA--------------BBA----B------AAAAA
YGRINHLDIPFHQLRDRVTYIPQESFFFSGSIIENLTFSLDTPPSFERIMEVCDAVQLGTFINQQPLRYDTILEEGGTNISGGQRQRLAI

ARALLKDADILILDEATSGLDTLLEHAILDYLINLEEKTLIFIAHHLSIAKACNQILVLNDGKLVEQGTHSDLRYNQGVYQQLWDI
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AAAAAAAA-AABBAA----AAAAAAAAAAAAAAAAAAAAAAAAAAA-AAAAAAAAABB-----ABB-------B-----AAB----
ARALLKDADILILDEATSGLDTLLEHAILDYLINLEEKTLIFIAHHLSIAKACNQILVLNDGKLVEQGTHSDLRYNQGVYQQLWDI
```

B.)

```
MHNNNWRDHSSVYSQQHQKFYRWVLYPVILFLFLVSLFLAFAKKEVVVRVPAQLTATKIDKLQVPLEANIKVNNLKENKEVKKGEVLVMF
---------------AAAAAAABB---ABBBBAAAAAAAAAA--ABBBB------A-A-------AA--A--------AAAA--ABBBBA
---------------AAAAAAABB---ABBBBAAAAAAAAAA--ABBBB------A-A-------AA--A--------AAAA--ABBBBA
MHNNNWRDHSSVYSQQHQKFYRWVLYPVILFLFLVSLFLAFAKKEVVVRVPAQLTATKIDKLQVPLEANIKVNNLKENKEVKKGEVLVMF

DTSVLQNEKEQFEQENTSLEDQKKAAQLFIESIAKEQNLFEDEDEFGYSNQLKSLLLEKETQNTEQVLATTKQKIAVLIETKKKNNYTLQ
----------AAAA----AAAAAAAAAAAAAAAAAAA-------------AAAAAAAA----AAAAAAAAAAAABBBAAAA--------A
----------AAAA----AAAAAAAAAAAAAAAAAAA-------------AAAAAAAA----AAAAAAAAAAAABBBAAA-----BBBB-
DTSVLQNEKEQFEQENTSLEDQKKAAQLFIESIAKEQNLFEDEDEFGYSNQLKSLLLEKETQNTEQVLATTKQKIAVLIETKKKITITFK

NITEQLVRGTLKSPSTGIVHLNEEVTGQIDVSKGTVLAEIYPKQKESQLMFTALLPADEVTRIKVGMDVHFKLDKKGVASQTIEGTLKEI
-AAAAAAA---------BBBB-------BB-----BBBBA-------AAAAAA------AABBB----AAAA--------BBAA--AAAA
-
I

SETSMTTEQGTFYTIKGTLQSSKYFNNRYGNTGEVSLIIGKKSYWQQIKDVLLNQE
------B----BBBBB------------------BBBBB------AAAAAAA----
```

Figure 6.4. Predicted secondary structure of the proteins in the piscicolin 126 operons found in *C. maltaromaticum* UAL26 and JG126. A.) PisT, B.) PisE. The first two lines in each set of four are the protein sequence and predicted secondary structure for *C. maltaromaticum* JG126, while the bottom two lines are the predicted secondary structure and protein sequence for *C. maltaromaticum* UAL26. A) α-helix; (B) β-sheet; (-) turn element. Differing residues are underlined, different predicted secondary structure is highlighted in bold.

6.3.3. Three-dimensional modelling of the piscicolin 126 structural peptide

The RMSD data showed that over 5 ns of simulation time the structure of piscicolin 126 solvated in TFE was stabilized after approximately 1 ns, and showed little structure variation over the remaining 4 ns of simulation time (Figure 6.5).

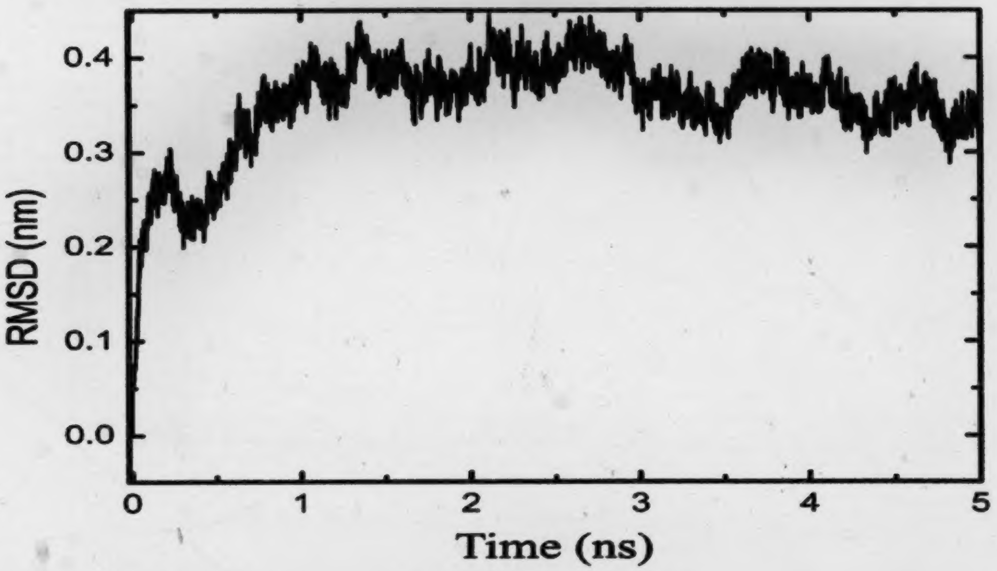

Figure 6.5. Root-mean-square deviation (RMSD) of the piscicolin 126 peptide solvated in trifluoroethanol fitted onto its original structure. Obtained from Dr. Kamaljit Kaur (Department of Pharmacy, University of Alberta).

The 3-D structure of the piscicolin 126 structural peptide is shown in Figure 6.6. The side chain model shows the side chain groups of each amino acid in the peptide, including a hydrophobic region of β-sheet structure at the N-terminus (light blue ribbon) and an α-helical coil in the central region of the peptide (red coil). The disulphide bridge is represented by two yellow spheres located after the region of β-sheet structure. In the backbone model, the N-terminal region shows a 3-stranded anti-parallel β-pleated sheet held together by the disulphide linkage, followed by the α-helical region.

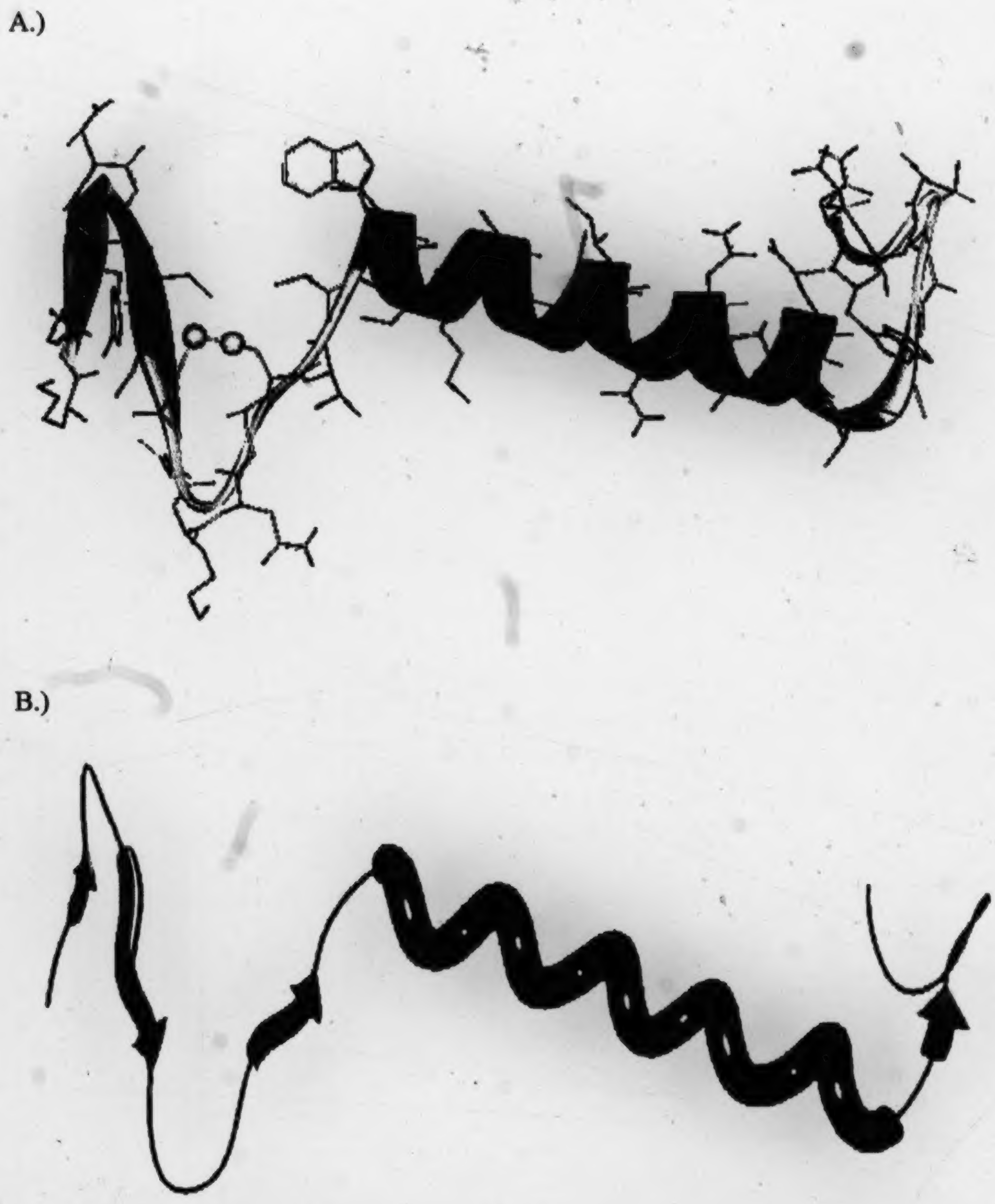

Figure 6.6. Minimized average structure of the piscicolin 126 peptide solvated in TFE over the last 1 ns of MD simulation. The N-terminus of the peptide is on the left side of the diagram, the C-terminus on the right. A.) side chain model; B.) backbone model. Arrows indicate regions of β-sheet structure, coils indicate regions of α-helical structure. Obtained from Dr. Kamaljit Kaur (Department of Pharmacy, University of Alberta) and Dr. John C. Vederas (Department of Chemistry, University of Alberta).

6.3.4. Construction of the plasmids pLG26PISTC and pLG26PISEC, and transformation of *C. maltaromaticum* UAL26

The transformation of *C. maltaromaticum* UAL26 with pLG26PISTC plasmid DNA resulted in the growth of 15 colonies. While all of the colonies contained the pMG36e plasmid, none were observed to have the engineered insert containing the functional *pisT* gene. The transformation of *C. maltaromaticum* UAL26 with pLG26PISEC plasmid DNA resulted in no growth of colonies.

6.4. Discussion

The discovery of 34 differences in the sequence of the piscicolin 126 operon in *C. maltaromaticum* UAL26 compared with the sequence of the piscicolin 126 operon in *C. maltaromaticum* JG126 is important because it may lead to an understanding of the difference in bacteriocin production between *C. maltaromaticum* UAL26 and JG126. The frameshift in the *pisE* gene in *C. maltaromaticum* UAL26 truncates the PisE protein at 181 of 326 residues. Additionally, there were 13 non-silent single bp differences, of which 10 are located in the *pisT* transporter gene. Only 2 non-silent differences resided in genes not involved in bacteriocin export; one in the immunity gene, and the other in the histidine kinase gene. This suggests that the functionality of transport in *C. maltaromaticum* UAL26 may be different than that in *C. maltaromaticum* JG126. Transport functionality may also affect bacteriocin processing (cleavage of the leader peptide) of both piscicolin 126, and carnobacteriocin BM1. Export of the induction peptide may also be hindered by the altered transport genes. Detection of bacteriocin by spot-on-lawn assay is achievable in *C. maltaromaticum* UAL26 grown at 25°C by supplementing growth media with synthetic induction peptide (Chapter 5, this thesis), but does not reach the bacteriocin titre produced by a culture of *C. maltaromaticum* UAL26 grown at 25°C. It is possible that the bacteriocins and the induction peptide are not being properly processed and exported at full efficiency in *C. maltaromaticum* UAL26 when grown at 25°C. In this case, it is hypothesized that some bacteriocin is able to exert antibacterial activity against *L. monocytogenes* ATCC 43256. As the bacteriocin titre of a culture of *C. maltaromaticum* UAL26 supplemented with induction peptide is lower than the bacteriocin titre produced by *C. maltaromaticum* UAL26 when grown at 15°C or that of *C. maltaromaticum* JG126 grown at 25°C, either unprocessed bacteriocin has some minor antibacterial activity against the indicator organism, or the transport proteins are able to properly process and transport bacteriocin at reduced efficiency. Differences in the sequences of the transport and accessory genes have also been shown in *Leuconostoc mesenteroides* Y105 and *L. mesenteroides* FR52 (Aucher *et al.*, 2004). Both strains of *L. mesenteroides* produce the bacteriocins mesentericin Y105 and B105. Although differences in the sequence of the dedicated bacteriocin transport genes were found, either strain is capable of maturation and export of both bacteriocins.

The differences in the amino acid sequence of the proteins of the piscicolin 126 operons found in *C. maltaromaticum* UAL26 and JG126 generally do not suggest a large change in their physical or chemical characteristics. Most of the changes observed involve either a change in polarity of the amino acid (polar to nonpolar or vice-versa), and increase in size (from tiny to small or small to average), or aromaticity (aliphatic to aromatic, or vice-versa). Amino acid residue differences numbers 4 and 5 in Table 6.4 are inverted, suggesting that these two residues may be necessary together in that region of the PisT protein. The two residue differences of greater importance are numbers 12 and 13 (Table 6.4). In difference number 12, there is a hydrophobic, polar, positive charge at this residue instead of only the polar trait. Gain of a positive charge could affect tertiary protein structure, causing a difference in protein folding. Difference number 13 is a change from a proline residue (a cyclic amino acid commonly found in turn elements in protein secondary structure) to threonine, a hydrophobic and polar residue. This difference may also affect secondary structure and folding of the PisT protein.

Hydropathy plots show some differences in hydrophobicity in the PisT and PisE proteins from *C. maltaromaticum* UAL26 and JG126. Most of the peaks that are different showed only a slight reduction or increase in hydrophobicity. The only notable changes in hydrophobicity were those found in the last 7 amino acids in the truncated PisE transport accessory protein in *C. maltaromaticum* UAL26, and the 145 additional amino acids present in the non-truncated version found in *C. maltaromaticum* JG126. Lack of difference in hydropathy suggests that the proteins may be similar in tertiary structure, regardless of amino acid difference.

Prediction of secondary structure showed some minor differences in predicted folding of the proteins of the piscicolin 126 operon that have differences in amino acid sequence. However, the differences observed are four amino acids or less in length. This suggests that secondary structure of the protein is unlikely to differ, as a length of only four amino acids is unlikely to constitute any large change in α-helix or β-sheet structure.

It is hypothesized that the *pisE* gene in *C. maltaromaticum* UAL26 is most likely to result in different hydropathy and secondary structure of the PisE transport accessory protein. The PisT protein also contained some differences, but showed less potential alteration in hydropathy or secondary structure than the PisE protein. The PisI immunity and PisK histidine kinase proteins may also be different from those in *C. maltaromaticum* JG126, but the results from amino acid similarity, hydropathy, and secondary structure prediction imply that these differences are far less likely to alter protein conformation than those found in the PisE accessory protein.

Attempts to create a strain of *C. maltaromaticum* UAL26 with a plasmid that contained the *pisT* or *pisE* gene from *C. maltaromaticum* JG126 were unsuccessful. This was similar to the attempts to create transformants of *C. maltaromaticum* UAL26 in Chapters 4 and 5 of this thesis. Transformation of *C. maltaromaticum* UAL26 with these elements of the piscicolin 126 operon from *C. maltaromaticum* JG126 did not result in the isolation of viable transformants which carried the plasmid with the correct insert. This prevented evaluation of the effects on bacteriocin production of these genes in *C. maltaromaticum* UAL26 grown at 25°C.

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7. GENERAL CONCLUSIONS

C. maltaromaticum UAL26 was isolated from ground beef, and was shown to produce antibacterial activity (Burns, 1987) Since this discovery, the goal has been the identification and characterization of the active compound(s) and the application of *C. maltaromaticum* UAL26 for use as a biopreservative in foods. For seventeen years, efforts to purify the antibacterial compounds were unsuccessful (Stiles, unpublished data). Although many common methods of isolating bacteriocins were attempted, none resulted in the isolation of enough bacteriocin at a quality pure enough for amino acid sequencing (Burns, 1987; Rosario, 2001).

Progress on the isolation of the bacteriocin from *C. maltaromaticum* UAL26 began with the evaluation of the differences between two assays used to measure bacteriocin activity. The deferred inhibition technique is more sensitive than the spot-on-lawn technique for the detection of antibacterial activity produced by *C. maltaromaticum* UAL26. Bacteriocin activity is observable when *C. maltaromaticum* UAL26 is grown on solid media, yet it is not detectable when the culture is grown at 25°C in liquid media. Upon observation that the diameter of the zone of bacteriocin activity from *C. maltaromaticum* UAL26 increased with decreased incubation temperature (Gursky *et al.*, 2002), evaluation of bacteriocin production in broth at decreased temperatures was attempted. Growth of *C. maltaromaticum* UAL26 at 4°C took significantly longer for the culture to reach maximum population, but bacteriocin was detectable by the spot-on-lawn assay. Growing *C. maltaromaticum* UAL26 at reduced temperatures (<19°C) increased the amount of bacteriocin that could be isolated from the supernatant, allowing both the isolation and characterization of the bacteriocin. A protocol was developed that allowed purification of sufficient quantities of a bacteriocin that had the same molecular mass as piscicolin 126. Amino acid sequencing of the bacteriocin revealed the presence of the YGNGVXC motif that is common in class IIa bacteriocins.

Probing the genome of *C. maltaromaticum* UAL26 revealed the presence of the piscicolin 126 and carnobacteriocin BM1 operons, although the carnobacteriocin BM1 peptide was never isolated from a broth culture of *C. maltaromaticum* UAL26. A possible reason that carnobacteriocin BM1 was not detected during the purification of piscicolin 126 could be that the indicator organism was *L. monocytogenes* ATCC 43256, a strain that is insensitive to carnobacteriocin BM1.

Piscicolin 126 was originally isolated from *C. maltaromaticum* JG126 (Jack *et al.*, 1996), and carnobacteriocin BM1 was originally isolated from *C. maltaromaticum* LV17B (Quadri *et al.*, 1994). The discovery of the genes encoding both bacteriocins in *C. maltaromaticum* UAL26 was welcomed, although with some despondency, because neither of these bacteriocins was a novel, uncharacterized compound. However, the phenomenon of temperature as a control for bacteriocin production had not been reported.

Piscicolin 126 and carnobacteriocin BM1 are class IIa bacteriocins. They have a spectrum of activity that is mainly limited to lactic acid bacteria and species closely related to LAB (Jack *et al.*, 1996). These bacteriocins are important because of their ability to inhibit strains of the foodborne pathogen *L. monocytogenes*. Both bacteriocins are present in the genomes of *C. maltaromaticum* UAL26 and JG126; however, the genes for production of piscicolin 126 are not present in *C. maltaromaticum* LV17 (unpublished data). In strains that contain the genetic elements for multiple bacteriocins, it can be difficult to attribute the cause of antibacterial activity to a particular bacteriocin.

Synergistic activity between bacteriocins can compound this problem (Limonet *et al.*, 2004).

Before the presence of the carnobacteriocin BM1 gene was discovered in the genome of *C. maltaromaticum* UAL26, the focus was to knock out piscicolin 126 production in *C. maltaromaticum* UAL26 by inserting an antibiotic resistance marker into the structural gene. This was attempted to determine if any other bacteriocin activity was being produced. If another bacteriocin was detected, it could be isolated and identified. If no other activity was found, the bacteriocin-negative strain could be used as a control organism in fundamental or applied food studies. Identification of the genes for carnobacteriocin BM1 in the genome of *C. maltaromaticum* UAL26 meant that two successive knockouts of the bacteriocin structural genes would be necessary. However, upon consideration of bacteriocin expression in *C. maltaromaticum* LV17B (Quadri *et al.*, 1994; Quadri *et al.*, 1997), it was hypothesized that carnobacteriocin BM1 might make use of the piscicolin 126 export machinery in a manner analogous to that of carnobacteriocin B2 in *C. maltaromaticum* LV17B. If the hypothesis was correct, a knockout of the piscicolin 126 transport or accessory gene should be sufficient to prevent the maturation and export of both bacteriocins.

Unfortunately, attempts at genetic manipulation of *C. maltaromaticum* UAL26 were unsuccessful. Transformation of *C. maltaromaticum* UAL26 with native plasmid DNA was successful, but attempts to transform it with plasmid DNA containing an insert encoding part of the piscicolin 126 operon always met with failure to isolate any colonies or growth of a very small number of background colonies containing the native plasmid without the insert. *C. maltaromaticum* UAL26 is apparently intolerant to attempts at insertion, replacement, or complement of elements of the piscicolin 126 operon. It remains possible that *C. maltaromaticum* UAL26 produces more than two bacteriocins.

Expression is one of the primary levels of control in bacteriocin production. Bacteriocins that are not always constitutively expressed will only be transcribed and translated when prompted to do so by a control mechanism. Piscicolin 126 production is controlled by this type of mechanism. Piscicolin 126 transcription in *C. maltaromaticum* UAL26 was not induced at 25°C and the expression of the piscicolin 126 operon is downregulated. Transcription of the other genes in the operon is detectable, but at lower levels than under temperatures permissive to increased bacteriocin production (15°C). At 15°C, levels of expression were comparable to that seen in *C. maltaromaticum* JG126. Carnobacteriocin BM1 may be transcribed at 25°C, but its antibacterial activity was not observed in spot-on-lawn assays.

Piscicolin 126 production can be induced in *C. maltaromaticum* UAL26 grown at 25°C by addition of supernatant containing the piscicolin 126 induction peptide or addition of synthetic piscicolin 126 induction peptide. Induction of the piscicolin 126 operon was possible using the spent supernatant from a culture of *C. maltaromaticum* JG126, but not with supernatant from a culture of *C. maltaromaticum* LV17. This suggested that there is no cross-induction between strains producing carnobacteriocins A or B2, and that carnobacteriocin BM1 production is not involved in induction of the piscicolin 126 operon.

In deferred inhibition assays, some antibacterial activity is observed when *C. maltaromaticum* UAL26 is grown at 25°C. The piscicolin 126 induction peptide may become sufficiently concentrated in solid media to induce transcription of the piscicolin

126 operon. Carnobacteriocin BM1 also may contribute to the size of the zones of inhibition observed when *C. maltaromaticum* UAL26 is grown on solid media.

C. maltaromaticum UAL26 and JG126 come from geographically distant locations. *C. maltaromaticum* UAL26 was isolated from vacuum packaged ground beef in Edmonton, Alberta, Canada (Burns, 1987), and *C. maltaromaticum* JG126 was isolated from spoiled ham in Victoria, Australia (Jack *et al.*, 1996). Both strains contain the same bacteriocin operons, but they have dramatic differences in the control of bacteriocin production. The genetic differences between the two strains include thirteen non-silent base pair differences and a single base pair deletion causing a frameshift in the *pisE* transport accessory gene. Although it is unknown which strain evolved from the other, it is plausible that the piscicolin 126 operon in *C. maltaromaticum* UAL26 was originally identical to that found in *C. maltaromaticum* JG126, but developed a truncation of the *pisE* transport accessory gene and other mutations in the operon during evolution. It is also possible that *C. maltaromaticum* JG126 developed an insertion of a single bp into its *pisE* transport accessory gene (extending the translated length of the protein) and other mutations in the operon.

It would be interesting to compare the genetic differences found in *C. maltaromaticum* UAL26 to other strains isolated from various food sources in different parts of the world. *C. maltaromaticum* strains that produce piscicolin 126 could be isolated from samples of vacuum packaged ground beef and spoiled ham, collected from around the world to see how these strains compare in the sequence of the piscicolin 126 operon with *C. maltaromaticum* UAL26 and JG126.

The phenomenon of temperature control remains unexplained at the biochemical level, yet elucidation of its ultimate cause may prove difficult due to the arduousness of genetic manipulation of the piscicolin 126 operon in *C. maltaromaticum* UAL26.

The genetic differences found between the piscicolin 126 operons in *C. maltaromaticum* UAL26 and JG126 may be related to the reduced bacteriocin activity observed when *C. maltaromaticum* UAL26 is grown at 25°C. Further evaluation of these differences and how they are related to the cause of reduced bacteriocin expression is the primary goal of further research with *C. maltaromaticum* UAL26. Specifically, evaluation of the *pisE* transport accessory and *pisT* ABC-transporter genes and their translated proteins would be the most obvious choice of a starting point. Additionally, evaluation of the relationship between carnobacteriocin BM1 and the piscicolin 126 transport machinery is of interest. Sequencing of the region surrounding the carnobacteriocin BM1 structural gene would reveal the presence of other genes in this operon in *C. maltaromaticum* UAL26. It is hypothesized that the lack of detection of bacteriocin activity in *C. maltaromaticum* UAL26 cultures grown in broth at 25°C may be due to differences in PisE and PisT protein folding, association, and substrate specificity. Conformational change in the transport proteins (due to differences in amino acid sequence) could prevent recognition of bacteriocin structural and induction peptides, thus preventing their maturation and export. Additionally, altered ability of the histidine kinase and response regulator to recognize the induction peptide in the extracellular media and activate piscicolin 126 transcription could interfere with induction of bacteriocin production. Genetic engineering of the *pisE* and *pisT* genes between *C. maltaromaticum* UAL26 and JG126 might explain the phenomenon of reduced bacteriocin activity produced by *C. maltaromaticum* UAL26 grown at 25°C when supplemented with synthetic induction peptide. Transcriptional analysis of the genes in the piscicolin 126 operon in *C. maltaromaticum* UAL26 could explain the differences in levels of gene expression, and the absence of piscicolin 126 structural gene expression when *C. maltaromaticum* is grown in broth at 25°C. The phenomenon of temperature control of piscicolin 126 expression in *C. maltaromaticum* UAL26 may be directly related to the differences found in the piscicolin 126 operon between *C. maltaromaticum* UAL26 and *C. maltaromaticum* JG126. These two effects could explain why full bacteriocin titre and transcription is observed in *C. maltaromaticum* UAL26 at 15°C, while reduced bacteriocin activity and full bacteriocin transcription at 25°C is detected under induced conditions, and no activity or transcription of the piscicolin 126 structural gene is observed in uninduced cultures grown in broth at 25°C. Clearly, the mechanism of control of piscicolin 126 production in *C. maltaromaticum* UAL26 is different and more complex than that in *C. maltaromaticum* JG126.

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APPENDIX

Comparison of the piscicolin 126 operon in *C. maltaromaticum* UAL26 (U) with the piscicolin 126 operon in *C. maltaromaticum* JG126 (J).

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J      #1  ATCGATAAAA TTTTTCATTA ACTAATAAAC AGATGTGCTG TTTTTTTGTA TAAAAATGTT TAAAACGATA
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J     #71  ATGTTTATTT AGAAAATTTG ATATCAAATA GTTCAAGGAT GATAATAGGA TTCACGATGT TGATAATATT
           ............................................................................

J    #141  AGCCTGCCTA AAGATACTGT GACATAAACA AAAAAATACA TTAAAAAAGA GAATGGGACA AAAGTCATAA
           ............................................................................

J    #211  AATAGCAAAA AAGCATGAAA TCCATTATTA CAAATGCTGA TTTCACACTA TTTTTTAATA GGTTGCTATT
           ............................................................................

J    #281  TACTAGAAAA TGGAGTTTTG TCCCACACTC TTTTTATTTT TTTATAAAAT AGATTAAATA GTTATTAGTA
           ............................................................................

J    #351  TTTTAAAAAA ATTCAGTTTA AAATTTGAAA TGACTACAAA TTAAAATGAG TGTTTAATTG AGTTTAGTAA
           ............................................................................

J    #421  AAAAATACTA TAATTATTTA TGGTACAAGA TAGTATTCAA ATGTAACTAT ACGAAATTTA TAAATAAAGA
           ............................................................................

J    #491  TGTTTTTTTT AGCACAATAT GTTACATTAT TTATGTGAGC AAGTTCGATA AAAGTTCGTC TCCCTAAAGT
           ............................................................................

J    #561  GATATAAAAC ACTTGAAGCT ATCTAAATAT TCATAAGTTT AAAAAGCTAT TATAACTTTT TCTTCTGAAA
           ............................................................................

J    #631  AATAATCAAA GGAAATTTTT ACGGCATCGC CCATGTGCTC GGGTAAGGGT CCTATAATAA GTGGGATACG
           ............................................................................

J    #701  CTAAATTTTT CCGTCTGTAA AGTTTAGAAG AGATTATCAG ACTAGCGATG CATGATGCCT GTTAGGCGGC
           ............................................................................

J    #771  TAATGTTCAG CGAAACCTTA ATAGCATGAC TATGAACGTA GATGTCTAAG TGCCGATATG CTTGGACAGG
           ............................................................................

J    #841  GGTTCGACTC CCCTCGTCTC CATATTGTGG TTATTTTAGA TGATAACCTA AAAATGAAAC CTATTTAAAA
           ............................................................................

J    #911  CGTTGATTTA ACAACGATTT AAATAGGTTT TTTTGTTTTA GGGAAATTAA AATAATAGTT AGTTTTAATT
           ............................................................................

J    #981  ATTTCAGTAC ACTAATTGTA CACTGAAATC AAATTTAATA ATTTCCATAA AAATTCTATT TTTGTTATTA
           ............................................................................
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J   #1051  GTGAATTGAC AGAATCCGAT TCACTTTTTT CTAATCTGTT TAAAATACCT TCATAAATAG TTAAACTTGT
U >#1071>                         TCACTTTTTT CTAATCTGCT TAAAATACCT TCATAAATAG TTAAACTTGT
           ............................................................................

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J   #1121  AATAATTTAC TATGACCTGA TCATTTTGAC GCATATTTAC TGGCATTATT TTCTTAACTA TTTTTATGAC
U   #1121  AATAATTTAC TATGACCTAA TCATTTTGAC GCATATTTAC TGGCATTATT TTCTTAACTA TTTTTATGAC
           ............................................................................

                              ◆
J   #1191  TAGTCGTAAA AATTTATAAG CCGATTTAAA GTAAATAGAT CAAATTTAAT TAAGATAGTG GGAGTTATAT
U   #1191  TAGTCGTAAA CTTTTATAAG TCGATTTAAA GTAAATAGAT CAAATTTAAT TAAGATAGTA GGAGTTATAT
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                      ◆◆         ◆                                         ◆
J   #1261  AGGTATATGC AGTACTAATA TCCATATCTA ATATTGGAGA TAGAAGTCAA CTCTTTAAGT TTTTTTGATT
U   #1261  AGGTATATGC AGTACTAATA TCCATATCTA ATATTGGAGA TAGAAGTCAA CTCTTTAAGT TTTTTTGATT
           ............................................................................
                                         PisI

J   #1331  GATAGTCAGA TAAAGTAACG CCATCATTTC TGATTGCTTT AGAAATATCT AAGTTCATTC GACTTAAAAT
U   #1331  GATAGTCAGA TAAAGTAACG CCATCATTTC TGATTGCTTT AGAAATATCT AAGTTCATTC GACTTAAAAT
           ............................................................................


J   #1401  CAGAGGAACA GAAGCGCCCT TTTGTTTTAA CTCTTCAAGA TAATTTTCTA ACACTTTTTT TAGCGATTCG
U   #1401  CAGAGGAACA GAAGCGCTCT TTTGTTTTAA CTCTTCAAGA TAATTTTCTA ACACTTTTTT TAGCGATTCG
           ............................................................................

                             ◆
J   #1471  TTGTCTAAGT CTGTTTTTAA ATCATCTAAT AAATCAGTGA TAATGTTTTC TGCCTGATTT GATCGTTCTT
U   #1471  TTGTCTAAGT CTGTTTTTAA ATCATCTAAT AAATCAGTGA TAATGTTTTC TGCCTGATTT GATCGTTCTT
           ............................................................................


J   #1541  TTCCTCCAGA AAACCATTTT AACTTACCCA TAAAATATCA CTCCTTAATT TTATATATTT ATGATTTTAG
U   #1541  TTCCTCCAGA AAACCATTTT AACTTACCCA TAAAATATCA CTCCTTAATT ATATATATTT ATGATTTTAG
           ............................................................................

                                                                  ◆
J   #1611  TTGATAGTAT AAGTTTCTTT ACATCATGTT GAAACTAGCC TATTTTTGTT AGTATAACAT AATTCCTAAG
U   #1611  TTGATAGTAT AAGTTTCTTT ACATCATGTT GAAACTAGCC TATTTTTGTT AGTATAACAT AATTCCTAAG
           ............................................................................


J   #1681  ACTATAAAAA AATATTTTTA TATATTTATT TTTGTTTTAA ATGTAACTTA CAAATATAAA GACCCATCTA
U   #1681  ACTATAAAAA AATATTTTTA TATATTTATT TTTGTTTTAA ATGTAACTTA CAAATATAAA GACCCATCTA
           ............................................................................


J   #1751  TGTTATGTCA CAGCATTGAT GCGTATCATC TTTTTTACAA GATAAAAAAT AAGAGACTTT AATTATCCTT
U   #1751  TGTTATGTCA CAGCATTGAT GCGTATCATC TTTTTTACAA GATAAAAAAT AAGAGACTTT AATTATCCTT
           ............................................................................
                                                                               PisA

J   #1821  TGTTCCAACC AGCGGCTCCA CCTGTAGTCA AATTTGCTGC TGCATTGTTT CCTATAATCC CAATAGCTTT
U   #1821  TGTTCCAACC AGCGGCTCCA CCTGTAGTCA AATTTGCTGC TGCATTGTTT CCTATAATCC CAATAGCTTT
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J  #1891  GCTCCAATCT ACAGTACAAC CATTTTTATT ACAGGAAACG CCATTTCCGT AATACTTACC TCCTGTAGTT
U  #1891  GCTCCAATCT ACAGTACAAC CATTTTTATT ACAGGAAACG CCATTTCCGT AATACTTACC TCCTGTAGTT
          ............................................................................
                                       PisA

J  #1961  AGTTGCATTT CTTTAACGCT AAGTTCTTTA ACAGTTTTCA TGCGTAAACA CGCTCCTTTT TAAAATATTT
U  #1961  AGTTGCATTT CTTTAACGCT AAGTTCTTTA ACAGTTTTCA TGCGTAAACA CGCTCCTTTT TAAAATATTT
          ............................................................................

J  #2031  TTTACAACAT GCTGACTGTA TCACATCTTT TTTACTTATA GATTTTTTAT TCCTGAACTA TCACGATATT
U  #2031  TTTACAACAT GCTGACTGTA TCACATCTTT TTTACTTATA GATTTTTTAT TCCTGAACTA TCACGATATT
          ............................................................................

J  #2101  ATTCATGAAC TTATAAAGTT AATTGATTGT AACATCCAAG AATCATTTAC TAGTGCTTCA GGAATGTTTT
U  #2101  ATTCATGAAC TTATAAAGTT AATTGATTGT AACATCCAAG AATCATTTAC TAGTGCTTCA GGAATGTTTT
          ............................................................................

J  #2171  TCGTTTTATA ATTAAATGTA TGGCATACTA TCACTGTTAA AAAAATTGGA GGGAATAAAA ATGAACGATA
U  #2171  TCGTTTTATA ATTAAATGTA TGGCATACTA TCACTGTTAA AAAAATTGGA GGGAATAAAA ATGAACGATA
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J  #2241  AAAAATACTT GAAATTGAAA GAATGTTCAG AAAAAAAATT AAAACAAATA CAGGGAGGTA ATAAATCAGT
U  #2241  AAAAATACTT GAAATTGAAA GAATGTTCAG AAAAAAAATT AAAACAAATA CAGGGAGGTA ATAAATCAGT
          ............................................................................
                                        PisN

J  #2311  TATAAAAGGG AACCCCGCAT CTAATTTAGC TCAATGTGTT TTTTCTTTTT TTAAAAAATG CTAATGATTT
U  #2311  TATAAAAGGG AACCCCGCAT CTAATTTAGC TCAATGTGTT TTTTCATTTT TTAAAAAATG CTAATGATTT
          ............................................................................

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J  #2381  TTATTTTTGG TATAATTTAA TCAAGGAGGG ATTTTCTTGA TTATCATCGA TACAAAAGCG TACTTACTAA
U  #2381  TTATTTTTGG TATAATTTAA TCAAGGAGGG ATTTTCTTGA TTATCATCGA TACAAAAGCG TACTTACTAA
          ............................................................................

J  #2451  GAATGTTGCT AAATATTTTT ATTTTGTATT TCCTAATTCT ATTTATTACT AAAGATGAAG CTAAACCCAG
U  #2451  GAATGTTGCT AAATATTTTT ATTTTGTATT TCCTAATTCT ATTTATTACT AAAGATGAAG CTAAACCCAG
          ............................................................................
                                        PisK

J  #2521  TAAATATGTA AAGATACTTG TACTTATCCC TATTACCTTT TTTGTGGAAT TATTCACTGA TTTTTCTGAT
U  #2521  TAAATATGTA AAGATACTTG TACTTATCCC TATTACCTTT TTTGTGGAAT TATTCACTGA TTTTTCTGAT
          ............................................................................

J  #2591  TTGGTACCGA TGCTTAGCAG TTATTTTTTA TTGAAAGAGA AAGGAAAAAA GGAAATTATA CTTTTAAATA
U  #2591  TTGGTACCGA TGCTTAGCAG TTATTTTTTA TTGAAAGAGA AAGGAAAAAA GGAAATTATA CTTTTAAATA
          ............................................................................

J  #2661  AACTTATTCT ATGTTTAATT GTAAGCTACA GTATATCAAT CATTAGTTCA TTGATGATGG TAAACAAATT
U  #2661  AACTTATTCT ATGTTTAATT GTAAGCTACA GTATATCAAT CATTAGTTCA TTGATGATGG TAAACAAATT
          ............................................................................
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J   #2731  ATTTGGAGGT ATTAATGACT ACACCTATGT AACTGTACAA ATTATTTTTG AATGTCTCTT GGTAACTCTA
U   #2731  ATTTGGAGGT ATTAATGACT ACACCTATGT AACTGTACAA ATTATTTTTG AATGTCTCTT GGTAACTCTA
           ............................................................................

J   #2801  TTTATTTTGT TATACAAAAA GCTAGGAATT CAGGAAATCA TTGTAAAGTA TAGTTCTATT ACAACTTTTA
U   #2801  TTTATTTTGT TATACAAAAA GCTAGGAATT CAGGAAATCA TTGTAAAGTA TAGTTCTATT ACAACTTTTA
           ............................................................................
                                       PisK

J   #2871  TGTGCATGAT TTACTTATGG GGAATTTCTT TTTTCGTATC TTATACAGCT CATCAATATA AAGTATTTGA
U   #2871  TGTGCATGAT TTACTTATGG GGAATTTCTT TTTTCGTATC TTATACAGCT CATCAATATA AAGTATTTGA
           ............................................................................

J   #2941  TCAATTCATT ATGGGTGTGT CAGTTTTCCT TGTCGTGCAG ATTATATTTA TTTGCATTTT AGGAGTTCAA
U   #2941  TCAATTCATT ATAGGTGTGT CAGTTTTCCT TGTCGTGCAG ATTATATTTA TTTGCATTTT AGGAGTTCAA
           ............................................................................
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J   #3011  ACAACTAAGA AGCAAAAAGA AAAGTTTGAA AAGCAGTTTA AAGAACAAGA ACTTATTCAT TTAAAAAAAT
U   #3011  ACAACTAAGA AGCAAAAAGA AAAGTTTGAA AAGCAGTTTA AAGAACAAGA ACTTATTCAT TTAAAAAAAT
           ............................................................................

J   #3081  ACACTGATCA GTTGGAACAA AGTCAAGAAA AGCTAAATAA ATTTAAACAT GATTATAAAA ACTTATTACT
U   #3081  ACACTGATCA GTTGGAACAA AGTCAAGAAA AGCTAAATAA ATTTAAACAT GATTATAAAA ACTTATTACT
           ............................................................................

J   #3151  CAGTTTAAAA GAGATATCTT TCTTAAAAAA AGACTCAGAT TTTTCGAAAC AAGTTCAAGC ATTAGATGAA
U   #3151  CAGTTTAAAA GAGATATCTT TCTTAAAAAA AGACTCAGAT TTTTCGAAAC AAGTTCAAGC ATTAGATGAA
           ............................................................................

J   #3221  TATTCTAACC ATTATCTTGC GAAAGATATA TCAAGTAACA GTCAACAATT AAAGAATATA AAAAATTCAT
U   #3221  TATTCTAACC ATTATCTTGC GAAAGATATA TCAAGTAACA GTCAACAATT AAAGAATATA AAAAATTCAT
           ............................................................................

J   #3291  ATTTGAAAAG CCTTTTTATT TCAAAAATAT ATCAAGCCAA TGAGCATGAT CTTGATATTA AATTGGAATG
U   #3291  ATTTGAAAAG CCTTTTTATT TCAAAAATAT ATCAAGCCAA TGAGCATGAT CTTGATATTA AATTGGAATG
           ............................................................................

J   #3361  TAATCAAACT ATTGAACTAA TCCCTATTCC TATTTTTGAT TGTGTAAGAA TATTGGGGAT TGTTTTTGAT
U   #3361  TAATCAAACT ATTGAACTAA TCCCTATTCC TATTTTTGAT TGTGTAAGAA TATTGGGTAT TGTTTTTGAT
           ............................................................................
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J   #3431  AATGCAATTG AAGCGGCTAA AGAAAGTGAA GATCAAAAAA TCTCTCTTAT GATTTATCAA GACCCTTCAC
U   #3431  AATGCAATTG AAGCGGCTAA AGAAAGTGAA GATCAAAAAA TCTCTCTTAT GATTTATCAA GACCCTTCAC
           ............................................................................

J   #3501  AACTTGAATT TCTTCTCGAA AATAGTTGTA AAAAAACGAA TATTCCTATC AGAAAGTTGA TGGAGAAAGG
U   #3501  AACTTGAATT TCTTCTCGAA AATAGTTGTA AAAAAACGAA TATTCCTATC AGAAAGTTGA TGGAGAAAGG
           ............................................................................
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J   #3571  AATTTCAACA AAAGTAGACC ATAAAGGATT AGGATTGAAT ACGATTCAAG AAATAAATGA AAAGACCAAA
U   #3571  AATTTCAACA AAAGTAGACC ATAAAGGATT AGGATTGAAT ACGATTCAAG AAATAAATGA AAAGACCAAA
           ................................PisK....................................

J   #3641  AATATGTTTA TTCAATATAA AATGAAAGAC TGTACGTTCA CCACTAGCAT TATCCTTATT TGGTAAGAAA
U   #3641  AATATGTTTA TTCAATATAA AATGAAAGAC TGTACGTTCA CCACTAGCAT TATCCTTATT TGGTAAGAAA
           ........................................................................

J   #3711  GGAGCTATTA TGACTTACCC CATTATTATT TGCGAAGATC AAATCATTCA ACTACAGCAA ATTGAAACAA
U   #3711  GGAGCTATTA TGACTTACCC CATTATTATT TGCGAAGATC AAATCATTCA ACTACAGCAA ATTGAAACAA
           ........................................................................

J   #3781  TTATTCAAAA TTTCGCACTT TTTCATAGTG AGCTTTTTCA AATCAGTTTA AAAACACAAA GTCCCGATGA
U   #3781  TTATTCAAAA TTTCGCACTT TTTCATAGTG AGCTTTTTCA AATCAGTTTA AAAACACAAA GTCCCGATGA
           ........................................................................

J   #3851  AGTAGAAAAT TATTTGAAAA AGTTTAAACC TAAGCAGGGA ATTTACTTTT TAGATATTGA TTTAAATCAT
U   #3851  AGTAGAAAAT TATTTGAAAA AGTTTAAACC TAAGCAGGGA ATTTACTTTT TAGATATTGA TTTAAATCAT
           ................................PisR....................................

J   #3921  ACTACGAACG GAATTGATCT GGCAGAAAAA ATACGTGTAC AAGATGTCCA AGCAAAAATT ATTTTTATTA
U   #3921  ACTACGAACG GAATTGATCT GGCAGAAAAA ATACGTGTAC AAGATGTCCA AGCAAAAATT ATTTTTATTA
           ........................................................................

J   #3991  CAACGCATGA TGAAATGATT CCTTTAACTA TCAAAAGGCG TGTGGAAACA TTAGGATTTG TGATAAAAGA
U   #3991  CAACGCATGA TGAAATGATT CCTTTAACTA TCAAAAGGCG TGTGGAAACA TTAGGATTTG TGATAAAAGA
           ........................................................................

J   #4061  TCAACCTTTG GATAATTATC GGTCAGAAAT TGTCGAGTTA CTACTCCTTG CTCAACAAAG AATTGATGCG
U   #4061  TCAACCTTTG GA[illegible]ATTATC GGTCAGAAAT TGTCGAGTTA CTACTCCTTG CTCAACAAAG AATTGATGCG
           ........................................................................

J   #4131  GCAAAGGTTA GTTCAAATCA AGCATTTATT TTTTCTATCG GATCTCAAAC ATTTACTCTT GATTTACAAG
U   #4131  GCAAAGGTTA GTTCAAATCA AGCATTTATT TTTTCTATCG GATCTCAAAC ATTTACTCTT GATTTACAAG
           ........................................................................

J   #4201  ATATTTATTT TTTAGAACCT TCTAAGCTAC CCCATAGATT AACCCTATAT ACTAAAAGTG GACAATATGA
U   #4201  ATATTTATTT TTTAGAACCT TCTAAGCTAC CCCATAGATT AACCCTATAT ACTAAAAGTG GACAATATGA
           ........................................................................

J   #4271  ATTTTATGGA AAGTTAGTTG ATTTAGAAAA AAAATACACT ACTTTATCCA AAATTAATCG ATCCTGCTTA
U   #4271  ATTTTATGGA AAGTTAGTTG ATTTAGAAAA AAAATACACT ACTTTATCCA AAATTAATCG ATCCTGCTTA
           ........................................................................

J   #4341  GCGAATTTAA TTAATGCAAA AGAAGTTAAT TTTAAAACGC GTGCTGTTTA TTTTGAGTCT GACTTAGTAA
U   #4341  GCGAATTTAA TTAATGCAAA AGAAGTTAAT TTTAAAACGC GTGCTGTTTA TTTTGAGTCT GACTTAGTAA
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J  #4411  GGCTTTTTGC ATTAGGTAAG GCAAAGCAAA TTAAAGAAAA ATTAAATGAA CAGTAACTTA ATTTTGAAAG
U  #4411  GGCTTTTTGC ATTAGGTAAG GCAAAGCAAA TTAAAGAAAA ATTAAATGAA CAGTAACTTA ATTTTGAAAG
          ............................PisR............................................

J  #4481  GACCGCTGTA TCGTAATGAA ACAATTTACT CATATTCAGC AACAAGATGA AAAAGATTGT GGTGTAGCAT
U  #4481  GACCGCTGTA TCGTAATGAA ACAATTTACT CATATTCAGC AACAAGATGA AAAAGATTGT GGTGTAGCAT
          ............................................................................

J  #4551  GTATAGCAAT GATTTTAAAA CATTACAAAA CAGAAATTCC CATTCACAAA TTACGTGATT TTTCAGGAAC
U  #4551  GTATAGCAAT GATTTTAAAA CATTACAAAA CAGAAATTCC CATTCACAAA TTACGTGATT TTTCAGGAAC
          ............................................................................

J  #4621  TGACTTAGAA GGCACATCGG CTTTTGGATT AAAGAAAACG TTTGAAAAGT TATTTTTTGA TTGTCCTGCT
U  #4621  TGACTTAGAA GGCACATCGG CTTTTGGATT AAAGAAAACG TTTGAAAAGT TATTTTTTGA TTGTCCTGCT
          ............................PisT............................................

J  #4691  ATCCAAGCTG ATAACGATGT GTGGAAAGAA AAAGAATTAC CCCTACCTTT AATTGCGCAC ATCTTGATTG
U  #4691  ATCCAAGCTG ATAACGATGT GTGGAAAGAA AAAGAATTAC CCCTACCTTT AATTGCGCAC ATCTTGATTG
          ............................................................................

J  #4761  ATAATAGCTA TATGCACTAT GTAGTTGTCT ATAAGATAAA TGGAGAAACA TTATCTATTG ATGATCCTGC
U  #4761  ATAATAGCTA TATGCACTAT GTAGTTGTCT ATAAGATAAA AGGAGAAACA TTATCTATTG ATGATCCTGC
          ............................................................................
                                                      •
J  #4831  TAGGGGGAAA ATAAAAAAAA CAGTTGAAGA ATTTGCTAAA GAATGGACAG GTATTTTATT ATTACCTACT
U  #4831  TAGGGGGAAA ATAAAAAAAA CAGTTGAAGA ATTTGCTAAA GAATGGACAG GTATTTTATT ATTACCTACT
          ............................................................................

J  #4901  CCAAAAGATA GTTACGTCCC AAGTAAAGAA AAAGTTGCGA GAATAAGCTC TTTTTTACCA GTTATTTGGC
U  #4901  CCAAAAGATA GTTACGTCCC AAGTAAAGAA AAAGTTGCGA GAATAAGCTC TTTTTTTCCA GTTATTTGGC
          ............................................................................
                                                                       •
J  #4971  TGCAAAAAGG TTTTGTTTTT CACATTATAG TAGCATCAAT TTTTATTACT TTGTTCGGTA TAGGAAGTTC
U  #4971  TGCAAAAAGG TCTTGTTTTT CACATTATAG TAGCATCAAT TTTTATTACT TTGTTCGGTA TAGGAAGTTC
          ............................................................................
                      •
J  #5041  TTACTATTTT CAAGGAATTC TAGACTATTT TATTCCAAAT CATGCTCGTT CTACATTAAA TATTGTATCA
U  #5041  TTACTATTTT CAAGGAATTC TAGACTATTT TATTCCAAAT CATACACGTT CTACATTAAA TATTGTATCT
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J  #5111  GTCGGTTTAA TTGTTGTTTA TTTATTTCGT GTCATGTTTG AATATAGTCG AAATTATCTC ATAGTTATAT
U  #5111  GTCGGTTTAA TTGTTGTTTA TTTATTTCGT GTCATGTTTG AATATAGTCG AAATTATCTC TTAGTTATAT
          ............................................................................
                                                                            •
J  #5181  TGGGGCAACG AATGAGTATG GTGGTCATGC TTCAGTACTT TAAACATATA CTTTCCTTAC CAATGAATTT
U  #5181  TGGGGCAACG AATGAGTATG GTGGTCATGC TTCAGTACTT TAAACATATA CTTTCCTTAC CAATGAATTT
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J  #5251  TTTTGCTACT CGTAAATCAG GTGAAATCAT TTCACGTTTT CTTGATGCTA ATAAAATCAT TGATGCTTTA
U  #5251  TTTTGCTACT CGTAAATCAG GTGAAATCAT TTCACGTTTT CTTGATGCTA ATAAAATCAT TGATGCTTTA
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J  #5321  GCAAGTGCCA CTTTGTCGGT TTTTTTAGAT ATTGGGATGG TCTTAATAGT AGGGATTACG TTAGCCGTTC
U  #5321  GCAAGTGCCA CTTTGTCGGT TTTTTTAGAT ATTGGCATGG TCTTAATAGT AGGGATTACG TTAGCTGTTC
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J  #5391  AAAATGGCAT CTTATTTTTA ATTACATTGG CTTCATTACC TTTTTATCTA GTTTCTATTT TAGCTTTTGT
U  #5391  AAAATGGCAT CTTATTTTTA ATTACATTGG CTTCATTACC TTTTTATCTA GTTTCTATTT TAGCTTTTGT
          ...........................................................................
                                         PisT

J  #5461  TAAAAGCTAT GAAAAAGCAA ATCAAGAAGA AATGGTTGCT GGTGCAACAT TAAATTCTAG TATTATTGAA
U  #5461  TAAAAGCTAT GAAAAAGCAA ATCAAGAAGA AATGGTTGCT GGTGCAACAT TAAATTCTAG TATTATTGAA
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J  #5531  AGTCTTAAGG GGATTGAGAC TATTAAGGCA TACAGCGGTG AAGAAAAGGT TTATCATCAA GTCGATAGAG
U  #5531  AGTCTTAAGG GGATTGAGAC TATTAAGGCA TACAGCGGTG AAGAAAAGGT TTATCATCAA GTCGATAGAG
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J  #5601  AATTTGTTCA ACTAATGAAA AAGTCTTTTC GTACGGCGAC ATTAGATAAT GTTCAGCAAG GTATTAAACA
U  #5601  AATTTGTTCA ACTAATGAAA AAGTCTTTTC GTACGGCGAC ATTAGATAAT GTTCAGCAAG GTATTAAACA
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J  #5671  TGCTATTCAG TTAATCAGTA GTGCATTAAT TTTGTGGCTA GGTTCTTATT ATGTTATGGA AGGGTCGATT
U  #5671  TGCTATTCAG TTAATCAGTA GTGCATTAAT TTTGTGGCTA GGTTCTTATT ATGTTATGGA AGGGTCGATT
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J  #5741  AGCCTAGGCC AACTTATTAC TTATAATGCC TTACTTGTTT TTTTTACTGA TCCTTTGCAA AATATTATTA
U  #5741  AGCCTAGGCC AACTTATTAC TTATAATGCC TTACTTGTTT TTTTTACTGA TCCTTTGCAA AATATTATTA
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J  #5811  ACTTACAAGT GAAAATGCAG ACAGCCCAAG TTGCTAATAA ACGCTTAAAT GAAATTTTTG CTATCGAGCC
U  #5811  ACTTACAAGT GAAAATGCAG ACAGCCCAAG TTGCTAATAA ACGCTTAAAT GAAATTTTTG CTATCGAGCC
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J  #5881  TGAATCAACT AATCAACACC CTGACCGTGT TGTTACAAAT CAAACATTTC AACAGGGAAT CACCTTGGAA
U  #5881  TGAATCAACT AATCAACACT CTGAACGTGT TGTTATAAAT CAAACATTTC AAAAGGGAAT TACCTTAGAA
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J  #5951  GGGGTTTCAT TTTCTTACAA CATGAAAGCT CCAACGTTGA GCAATCTCTC TTGTACGATT CTACCACATA
U  #5951  GGGGTTTCAT TTTCTTACAA CATGAAAGCT CCAACGTTGA GCAATCTCTC TTGTACGATT CTACCACATA
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J  #6021  GCAAGGTTGC CTTAGTTGGT GTTAGTGGAT CTGGAAAATC TACCATGGCA AAATTACTAG TGAATTTTTA
U  #6021  GCAAGGTTGC CTTAGTTGGT GTTAGTGGAT CTGGAAAATC TACCATGGCA AAATTACTAG TGAATTTTTA
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J  #6091  TCCCCCATCT GAAGGAACAA TTCGTTATGG CCGTATCAAT CATCTAGATA TTCCTTTTCA TCAATTACGT
U  #6091  TCCCCCATCT GAAGGAACAA TTCGTTATGG CCGTATCAAT CATCTAGATA TTCCTTTTCA TCAATTACGC
          ....................................PisT....................................
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J  #6161  GACCGAGTAA CCTATATTCC TCAAGAATCA TTTTTTTTCA GTGGATCTAT CATTGAAAAT TTAACATTTA
U  #6161  GACCGAGTAA CCTATATTCC TCAAGAATCA TTTTTTTTTA GTGGATCTAT CATTGAAAAT TTAACATTTA
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J  #6231  GCTTGGATAC ACCACCTAGT TTTGAACGAA TAATGGAAGT TTGTGATGCA GTTCAATTGG GTACATTTAT
U  #6231  GCCTGGATAC ACCACCTAGT TTTGAACGAA TAATGGAAGT TTGTGATGCA GTTCAATTGG GTACATTTAT
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J  #6301  TAATCAACAA CCTTTACGTT ATGACACGAT ACTAGAGGAG GGTGGGACCA ATATTTCTGG TGGGCAACGT
U  #6301  TAATCAACAA CCTTTACGTT ATGACACGAT ACTAGAGGAG GGTGGGACCA ATATTTCTGG TGGGCAACGT
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J  #6371  CAAAGATTAG CTATTGCTCG TGCTTTATTG AAGGACGCCG ACATCCTAAT CTTAGATGAG GCGACCAGTG
U  #6371  CAAAGATTAG CTATTGCTCG TGCTTTATTG AAGGATGCCG ACATCCTAAT CTTAGATGAG GCGACCAGTG
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J  #6441  GACTGGATAC ACTCTTAGAA CATGCCATAT TAGACTACTT AATAAATTTA GAAGAAAAAA CCTTGATTTT
U  #6441  GACTGGATAC ACTCTTAGAA CATGCCATAT TAGACTACTT AATAAATTTA GAAGAAAAAA CCTTGATTTT
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J  #6511  CATTGCTCAT CATTTATCAA TAGCCAAAGC CTGTAATCAA ATTTTAGTAC TCAACGATGG AAAATTAGTA
U  #6511  CATTGCTCAT CATTTATCAA TAGCCAAAGC CTGTAATCAA ATTTTAGTAC TCAACGATGG AAAATTAGTA
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J  #6581  GAGCAAGGTA CGCACAGCGA TTTGCGATAT AATCAGGGAG TTTATCAACA GTTATGGGAT ATTTAAAAAA
U  #6581  GAGCAAGGTA CGCACAGCGA TTTGCGATAT AATCAGGGAG TTTATCAACA GTTATGGGAT ATTTAAAAAA
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J  #6651  GGAAGGCTAA ATTATGCATA ATAATAACTG GCGCGACCAC TCCAGTGTAT ATAGTCAACA GCATCAAAAA
U  #6651  GGAAGGCTAA ATTATGCATA ATAATAACTG GCGCGACCAC TCCAGTGTAT ATAGTCAACA GCATCAAAAA
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J  #6721  TTTTATCGTT GGGTGTTATA TCCTGTAATT CTTTTTTTGT TTTTGGTTAG TTTATTTTTG GCTTTTGCGA
U  #6721  TTTTATCGTT GGGTGTTATA TCCTGTAATT CTTTTTTTGT TTTTGGTTAG TTTATTTTTG GCTTTTGCGA
          ....................................PisE....................................

J  #6791  AAAAAGAAGT TGTCGTTCGG GTACCTGCTC AACTAACCGC AACTAAAATA GATAAACTAC AAGTACCACT
U  #6791  AAAAAGAAGT TGTCGTTCGG GTACCTGCTC AACTAACCGC AACTAAAATA GATAAACTAC AAGTACCACT
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J  #6861  GGAAGCAAAC ATCAAAGTAA ATAATCTTAA AGAAAATAAA GAGGTCAAAA AAGGAGAAGT TTTAGTGATG
U  #6861  GGAAGCAAAC ATCAAAGTAA ATAATCTTAA AGAAAATAAA GAGGTCAAAA AAGGAGAAGT TTTAGTGATG
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J   #6931   TTTGATACTA GTGTTTTACA AAATGAAAAG GAGCAATTTG AACAAGAAAA TACGTCTCTT GAAGACCAAA
U   #6931   TTTGATACTA GTGTTTTACA AAATGAAAAG GAGCAATTTG AACAAGAAAA TACGTCTCTT GAAGACCAAA
            ............................................................................
                                            PisE

J   #7001   AGAAAGCTGC GCAACTATTT ATTGAAAGTA TAGCTAAAGA ACAGAACTTA TTTGAAGACG AGGATGAATT
U   #7001   AGAAAGCTGC GCAACTATTT ATTGAAAGTA TAGCTAAAGA ACAGAACTTA TTTGAAGACG AGGATGAATT
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J   #7071   TGGATACAGT AATCAGCTAA AGAGTTTATT ACTCGAAAAG GAAACACAAA ATACAGAGCA AGTCTTGGCT
U   #7071   TGGATACAGT AATCAGCTAA AGAGTTTATT ACTCGAAAAG GAAACACAAA ATACAGAGCA AGTCTTGGCT
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J   #7141   ACAACCAAGC AAAAGATAGC CGTACTAATT GAAACCAAAA AAAAAAATAA CTATACCCTT CAAAATATAA
U   #7141   ACAACCAAGC AAAAAATAGC CGTACTAATT GAAA:CAAAA AAAAAAATAA CTATAACCTT CAAAATATAA
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J   #7211   CTGAACAGCT TGTACGAGGA ACATTAAAAT CTCCTTCAAC TGGAATAGTT CACTTGAATG AGGAAGTAAC
U   #7211   CTGAACAGCT TGTACGAGGA ACATTAAAAT CTCCTTCAAC TGGAATAGTT CACTTGAATG AGGAAGTAAC
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J   #7281   AGGCCAAATA GATGTATCAA AAGGAACGGT ACTTGCAGAA ATCTATCCAA AGCAAAAAGA ATCACAGCTA
U   #7281   AGGCCAAAGA GATGTATCAA AAGGAACGGT ACTTGCAGAA ATCTATCCAA AGCAAAAAGA ATCACAGCTA
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J   #7351   ATGTTTACTG CGCTATTACC TGCTGATGAA GTAACACGAA TAAAAGTAGG TATGGACGTT CATTTTAAAT
U   #7351   ATGTTTACTG CGCTATTACC TGCTGATGAA GTAACACGAA TAAAAGTAGG TATGGACGTT CATTTTAAAT
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J   #7421   TAGATAAAAA AGGTGTTGCA TCCCAAACAA TTGAGGGAAC TCTAAAAGAG ATTTCAGAGA CTAGTATGAC
U   #7421   TAGATAAAAA AGGTGTTGCA TCCCAAACAA TTGAGGGAAC TCTAAAAGAG ATTTCAGAGA CTAGTATGAC
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J   #7491   AACTGAACAA GGAACCTTCT ACACAATAAA AGGTACGTTA CAATCTTCAA AATATTTTAA TAATAGATAT
U   #7491   AACTGAACAA GGAACCTTCT ACACAATAAA AGGTACGTTA CAATCTTCAA AATATTTTAA TAATAGATAT
            ............................................................................

J   #7561   GGTATGACAG GGGAAGTTTC GCTCATTATT GGAAAAAAAA GCTATTGGCA ACAAATCAAA GATGTGTTGC
U   #7561   GGTATGACAG GGGAAGTTTC GCTCATTATT GGAAAAAAAA GCTATTGGCA ACAAATCAAA GATGTGTTGC
            ............................................................................

J   #7631   TAAATCAAGA ATAAAGTTTG TGTAATACCT GAAGGAATTA ATAAAATAAA ATTTAGATCA TGTCGTATGC
U   #7631   TAAATCAAGA ATAAAGTTTG TGTAATACCT GAAGGAATTA ATAAAATAAA ATTTAGATCA TGTCGTATGC
            ............................................................................

J   #7701   TATTGTATCT AAAAAAATAA TGTGTTGTTT TACTTTAAGT AAAACAACAC ATTATTAATC TAGAAATAGT
U   #7701   TATTGTATCT AAAAAAATAA TGTGTTGTTT TACTTTAAGT AAAACAACAC ATTATTAATC TAGAAATAGT
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J   #7771  ATCTTAAATT AAAAGTTAAG TGTTTTATTT TGACAATTAT TAACATAAAG ATTATGAGGA TAGGGTATAC
U   #7771  ATCTTAAATT AAAAGTTAAG TGTTTTATTT TGACAATTAT TAACATAAAG ATTATGAGGA TAGGGTAT
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J   #7841  CATTATTTTA GTAACTTACT ATTTAATTTT TTCATTGATA CATGACCAAA TATTAATCAA AATTCTTCGA
           ............................................................................

J   #7911  GTATCATTTG CATTTAAACT TGTTTAAATG CAGAGTAGAA TTTATTGAAA TTAAGTCAAT TAAGCTTACA
           ............................................................................

J   #7981  TAGATTTATA AATCTCATTA CACAGTCAAA GCTCCCCTCA TCTCCATATT TAGTACAAAC CAGAATAAAC
           ............................................................................

J   #8051  TAAAAGGGCT ATAAGGGTTG ATTTAAATGC GTTTTTAGCT CTTTTTGTTT TTATACGGGA GACTAGAAAA
           ............................................................................

J   #8121  AATAAAGAGT GCAAAATGTT TTAGGAAAAT GTATGAAATA TCGACTTAAA CAAGTAGAAC GCTAAACCAA
           ............................................................................

J   #8191  CTTCAGTAAA CGTAAAATTC CTTTGAAGCA CTTGAAAATA TTTGGCCCTT TTTCCAACAA TATTAATATT
           ............................................................................

J   #8261  TGCCGTTAAC GGGAAAAGGA ACAAAGTAGT TGT
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